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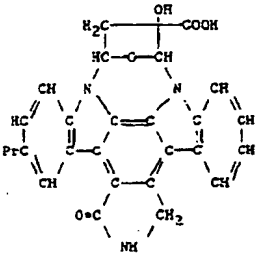

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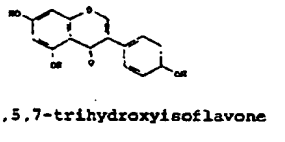

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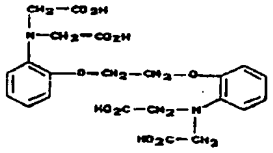

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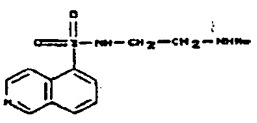

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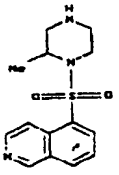

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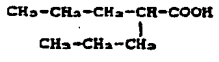

Valproic acid

(57) Abstract

The invention relates to a pharmaceutically acceptable prodrug which is a covalent conjugate of a pharmacologically active compound and an intracellular transporting adjuvant, characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity. The prodrug may be used in a technique for treating a condition or disease in a human related to supranormal intracellular enzyme (e.g. phospholipase and/or esterase) activity, whereby on administering it to a human having such condition or disease, the bond is broken in response to such activity, and the pharmacologically active compound accumulates selectively within cells having such supranormal intracellular enzyme activity. Exemplary conjugates are esters of the carboxylic function in the formula, with e.g. heptanoyl-sn-3-glycerophosphoryl-choline or octanoyl-sn-3-glycerophosphoryl-choline.

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PRODRUGS WITH ENHANCED PENETRATION INTO CELLS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a technique for treating a condition or disease in a human related to supranormal intracellular enzyme activity, and to a prodrug useful in such technique.

Ischemia, stroke, epilepsy, asthma and allergy are among the most frequently occurring disorders in humans.

Cerebrovascular disease, manifested e.g. as cerebral insufficiency, cerebral infarction, cerebral hemorrhage, or cerebral arteriovenous malformation, as well as stroke (ischemic lesions), constitutes the most common cause of neurological disability in developed countries.

Epilepsy affects about 2% of the population. No single drug controls all types of seizures, and different drugs or drug combinations are required for different patients.

Bronchial asthma is a reversible obstructive lung disorder. Asthma and allergies are very widespread diseases, especially in developed countries.

In spite of the obvious difference between the different diseases mentioned above, they are believed to be related to the phenomenon of cell hyperexcitation, in which cell membranes are broken down due to abnormal enzyme activity. Current pharmacological strategies are therefore aimed at inhibiting this degradative activity.

The cell damage occurring in ischemia may be secondary to the influx and/or intracellular release of Ca^{2+} ions (Siesjo and Smith, Arzneimittelforschung, 1991, 41(3A): 288-292). Similarly, calcium influx appears to play an important role in the genesis of epileptic seizures, although a significant portion of intracellular calcium arrives from intracellular stores, and current research suggests that calcium entry blockers may have anticonvulsant activity (see e.g. Meyer, 1989, Brain Res. Rev. 14: 227-243).

Drugs which are currently or potentially useful for treatment of calcium associated disorders include (1) calcium channel blockers, (2) drugs affecting calcium economy by modification of calcium intracellular storage sites, and (3) intracellular calcium chelating agents. Calcium channel blockers used in clinical practice are represented by Verapamil, Nifedipine and Diltiazem. The major toxicities associated with the use of such compounds involve excessive vasodilation, negative inotropy, depression of the sinus nodal rate, and A-V nodal conduction disturbances. Drugs affecting calcium mobilization/sequestration, like calcium channel blockers, exhibit rather narrow specificity. There is no intracellular calcium chelating agent available for clinical requirements. Existing calcium chelators such as EGTA-AM, EDTA-AM, and BAPTA-AM are available as complex molecules, the hydrophobic part of which could be digested by cellular noninducible esterase, thus causing accumulation of chelator intracellular space, which is, however, random and uncontrolled, being unrelated to cell activity.

It would be useful to be able to selectively target diseased cells characterized by enzyme hyperactivity, so as to introduce a pharmacologically active molecule in the form of a prodrug into the cell, whereby such hyperactivity would act on the prodrug, so that the pharmacologically active molecule accumulates in the diseased cells rather than in the active cells. A non-limiting example of such pharmacologically active molecule is a calcium chelating agent, which would have many advantages over drugs presently used for the treatment of calcium associated disorders.

Intracellular calcium is an important determinant for cell death in organ hypothermic preservation for transplantation, and may also be relevant in organs protection (toxicology). Additionally, calcium precipitated cell disintegration accepted as a key event on lymphocyte and killer cell mediated damaging of the target cells. Lymphocyte-target interaction leads to sustained elevation of the intracellular calcium level and causes a cascade of destruction. Prevention of calcium entry improved the result of liver cold storage in UW solution (Rajab et al, Transplantation, 1991, 51(5): 965-7). Myocyte injury can be produced by sensitized cytotoxic T lymphocytes in vitro and is calcium dependent (Woodley et al, Circulation, 1991, 83(4): 1410-8). Studies illustrate reduced rejection rates in organ transplant patients treated with calcium channel blockers (Weir, Am J. Med., 1991, 90(5A): 32S-36S). Thus it will be apparent that the present invention has potential use (in the embodiment employing a calcium chelator) in relation to these circumstances.

It will also be self-evident that a similar concept can be applied to the treatment of conditions or diseases other than those related to the intramolecular level of Ca^{2+} ions. By way of example, if the active entity incorporated in the prodrug molecule is a protein kinase inhibitor, after administration of the prodrug the inhibitor would be accumulated in a cell exhibiting abnormal proliferation, thus providing potentially an important tool for use in antitumor therapy.

SUMMARY OF THE INVENTION

In accordance with one object of the invention, there are provided prodrugs which selectively accumulate pharmacologically active compounds in hyperactivated cells. In accordance with another object of the invention, the pharmacologically active compound is released from the prodrug in response to enzyme activity in the targeted cells. In accordance with yet another object of the invention, the pharmacologically active compound, selectively accumulated in a cell characterized by a relatively raised level of enzyme activity therein, is trapped in the cell and therefore exhibits an enhanced desired activity therein.

The present invention accordingly provides in one aspect, a prodrug which is a covalent conjugate of a pharmacologically active compound and an intracellular transporting adjuvant, characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity.

In another aspect, the present invention provides a technique for treating a condition or disease in a human, related to supranormal intracellular enzyme activity, which comprises administering to a human having such condition or disease, a pharmaceutically acceptable cell membrane permeable prodrug, the prodrug being a covalent conjugate of a cell membrane impermeable pharmacologically active compound and an intracellular transporting adjuvant, characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity, such that the bond is broken in response to such activity, whereby the pharmacologically active compound accumulates selectively within cells having supranormal intracellular enzyme activity, the prodrug being administered in an amount effective for reducing the supranormal enzyme activity.

In yet another aspect, the invention provides use for the manufacture of a medicament for treating a condition or disease in a human related to supranormal intracellular enzyme activity, by selectively accumulating a cell membrane impermeable pharmacologically active compound within cells having such activity, of a pharmaceutically acceptable cell membrane permeable prodrug, which is a covalent conjugate of the pharmacologically active compound and an intracellular transporting adjuvant, and is characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity, such that the bond is broken in response to such activity.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be understood and appreciated more fully from the detailed description below, in conjunction with the drawings, in which:

Figure 1 is a graphical illustration of the effects of a compound, in accordance with an embodiment of the present invention, on intracellular free Ca^{2+} level in human lymphocytes;

Figure 2 compares recovery in Global Cerebral Ischemia in presence or absence of a compound in accordance with an embodiment of the invention;

Figure 3 illustrates the variation with dosage of pilocarpine induced epileptic events;

Figure 4 illustrates the protection against pilocarpine induced epileptic events afforded by a compound in accordance with an embodiment of the invention;

Figures 5 and 6 illustrate the protection against long-term alteration of certain cardiac functions, or shift of coronary vessels tone regulation, caused by pilocarpine, afforded by a compound in accordance with an embodiment of the invention;

Figure 7 illustrates the recovery of pilocarpine-damaged hearts in an Ischemia-Reperfusion model, when using a compound in accordance with an embodiment of the invention;

Figure 8 illustrates the protective effect in a metrazol minimum seizures test, afforded by a compound in accordance with an embodiment of the invention; and

Figures 9, 10 and 11 illustrate results of experiments in hypoxia-reperfusion cardiopathology.

DETAILED DESCRIPTION OF THE INVENTION

The pharmacologically active compound may be e.g. a pharmacologically active carboxylic acid, when the adjuvant may comprise (e.g.) at least one pharmaceutically acceptable alcohol which is selected from glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lyso-plasmalogens, lysophospholipids, lysophosphatidic acid amides, glycerophosphoric acids, lysophophatidal-ethanolamine, lyso-phosphatidylethanolamine and N-mono- and N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof. Exemplary of pharmacologically active carboxylic acids are branched-chain aliphatic carboxylic acids (e.g. valproic acid), salicylic acids (e.g. acetylsalicylic acid), steroidal carboxylic acids (e.g. lysergic and isolysergic acids), monoheterocyclic carbocylic acids (e.g. nicotinic acid) and polyheterocyclic carboxylic acids (e.g. penicillins and cephalosporins). While pharmacologically active carboxylic acids are particularly described herein, as exemplary of the active compounds which may be conjugated with an intracellular transporting adjuvant, the invention is not limited thereto. Thus, by way of further example, it is entirely within the concept of the present invention to conjugate therapeutically active nucleic acids (including RNA and DNA) or fragments thereof with an intracellular transporting adjuvant.

In a non-limitative embodiment, the prodrug according to the invention includes a calcium chelating agent, and may thus be of potential use for treating diseases or conditions which are

related to an unduly high level of intracellular Ca^{2+} ions. In a particularly preferred embodiment, the prodrug contains at least one covalent bond between the pharmacologically active compound and the intracellular transporting adjuvant, which covalent bond is scission-sensitive to intracellular enzyme activity, with the consequence that the greater part of the prodrug molecules will move freely in and out of normal cells without scission of such bond, whereas in the cells possessing the supranormal enzyme activity only, the scission-sensitive bond in a high proportion of prodrug molecules entering the cells will break, thus accumulating intracellularly, and trapping within the abnormal cell, the pharmacologically active compound, since the latter is cell membrane impermeable. Persons skilled in the art will appreciate in what manner the concept of the invention may be applied to conditions and diseases which are not necessarily related to an intracellular excess of calcium ions, so that in such other cases, the prodrug will incorporate an active compound which is not a calcium chelator but which will possess other desired pharmacological activity.

The prodrug which includes a calcium chelating agent is, e.g., a partially or totally esterified carboxylic acid, which is an ester of:

(a) a pharmaceutically acceptable chelating agent for calcium having the formula $(HOOC\text{-}CH_2\text{-})_2\text{-N-A-N-}(\text{-}CH_2COOH)_2$ where A is saturated or unsaturated, aliphatic, aromatic or heterocyclic linking radical containing, in a direct chain link between the two depicted nitrogen atoms, 2-8 carbon atoms in a continuous

chain which may be interrupted by 2-4 oxygen atoms, provided that the chain members directly connected to the two depicted nitrogen atoms are not oxygen atoms, with

(b) a C_{3-32} pharmaceutically acceptable alcohol containing 1-3 OH radicals (e.g. such a C_{3-6} alcohol, or e.g. a C_{7-32} secondary monohydric alcohol);

and salts with alkali metals of the partially esterified carboxylic acids, as well as acid addition salts of such of the esterified carboxylic acids as contain one or more potentially salt-forming nitrogen atoms.

The ester of the preceding paragraph may be one in which the linking radical A is a member selected from the group consisting of $-(CH_2CH_2)_m-$ where m = 1-4, in which 2-4 of the carbon atoms not attached to nitrogen may be replaced by oxygen atoms, and -CR=CR-O-CH_2CH_2-O-CR'=CR'-, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, complete an aromatic or heterocyclic ring containing 5 or 6 ring atoms, the ring completed by R-R being the same as or different from the ring completed by R'-R'.

In particular embodiments, the linking radical A may be, e.g., selected from $-CH_2CH_2-$ and $-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-$; or it may be e.g. -CR=CR-O-CH_2CH_2-O-CR'=CR'-, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, complete an aromatic or heterocyclic ring which is selected from the group consisting of furan, thiophene, pyrrole, pyrazole, imidazole, 1,2,3-triazole, oxazole, isoxazole, 1,2,3-

oxadiazole, 1,2,5-oxadiazole, thiazole, isothiazole, 1,2,3-thiadiazole, 1,2,5-thiadiazole, benzene, pyridine, pyridazine, pyrimidine, pyrazine, 1,2,3-triazine, 1,2,4-triazine, and 1,2-, 1,3- and 1,4-oxazines and thiazines, the ring completed by R-R being the same as or different from the ring completed by R'-R'. In a particularly preferred embodiment, the linking radical A is $-CR=CR-O-CH_2CH_2-O-CR'=CR'-$, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, completes the same or different rings selected from unsubstituted and substituted benzene rings, in which substituted benzene rings contain 1-4 substituents selected from the group consisting of C_{1-3}-alkyl, C_{1-3}-alkoxy, F, Cl, Br, I and CF_3, or a single divalent substituent which is $-O-(CH_2)_n-O-$ and n = 1-3.

It is presently preferred that the calcium chelating agent incorporated in the prodrug is selected from ethylene-1,2-diamine-N,N,N',N'-tetraacetic acid, ethylene-1,2-diol-bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid and 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

As mentioned above, C_{3-32}, e.g. C_{3-6}, alcohol referred to above contains 1-3 OH radicals. When 2 OH radicals are present, one of them may be esterified or otherwise derivatized, and when 3 OH radicals are present, either 1 or 2 of the OH radicals may be esterified or otherwise derivatized. Any carbon atoms in the esterifying or otherwise derivatizing group(s) are not counted for the purpose of the e.g. 3 to 6 carbon atoms which may be contained in the pharmaceutically acceptable alcohols. Thus, these alcohols may comprise, e.g., at least one member of

the group consisting of glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lysoplasmalogens, lysophospholipids, lysophosphatidic acid amides, glycerophosphoric acids, lysophophatidalethanolamine, lysophosphatidylethanolamine and the N-mono-C_{1-4}-alkyl, N,N-di-C_{1-4}-alkyl and quaternary ammonium derivatives of such of the foregoing as are amines. An example of a C_{7-32} secondary alcohol is 1-myristylmyristyl alcohol.

The person skilled in the art will appreciate that the prodrug of the present invention can be tailored in such a manner that the desired pharmacologically active entity is released by action of the enzyme known to be the source of enzyme hyperactivity in the condition or disease being treated. For example, membrane-associated calcium-independent plasmalogen-selective PLA_2 activity has been found to increase over 400% during two minutes of global ischemia ($P<0.01$), was greater than 10-fold (near to the maximum) after only five minutes of ischemia, and remained activated throughout the entire ischemic interval examined (up to 60 minutes), see Ford et al, J. Clin. Invest., 1991, 88(1): 331-5. These facts suggest attaching the pharmacological active entity to the 2-position in a glycerophosphoric acid derivative, and that use of a lysoplasmalogen may possibly be more effective as the intracellular transporting adjuvant, to which the active entity is attached covalently, than a lysophospholipid.

Any events (e.g. cytotoxic chemicals, physical stimuli and infective agents) causing damage of the cell membrane can trigger a cascade leading ultimately to a condition which mimics ischemia (Robbins et al, Pathological Basis for Disease, 1984, p. 10, W. B. Sanders Co.). The present invention will potentially be of use for protecting cells in these circumstances, by introduction of a calcium chelator intracellularly. In this connection, it is noted that the antitumor drug Adriamycin, which has been reported to inhibit Na-Ca exchange and to overload the sarcoplasm with calcium, could induce contractile heart failure; this would be consistent with the hypothesis that calcium overload, in absence of ischemia, can leave behind long-lasting contractile dysfunction (Kusuoka et al, J. Cardiovasc. Pharmacol., 1991, 18(3): 437-44).

As indicated above, the concept of the present invention is not restricted to the treatment of conditions or diseases related to the intramolecular level of Ca^{2+} ions, so that the materials used in practising the invention are not restricted to calcium chelators. Thus for example, the pharmacologically active compound may be e.g. an antiepileptic compound such as valproic acid. In this connection, it is contemplated that application of the present invention in this embodiment would enable a much lower effective dose of valproic acid to be used than is otherwise the case, thus potentially substantially reducing the occurrence of undesired side-effects. In principal, any of the range of alcohols, and examples thereof, mentioned above in connection with esterification of calcium

chelators may also be applied to the esterification of valproic acid in accordance with the concept of the present invention. In a non-limiting embodiment, valproic acid may be esterified with, e.g., 1-heptanoyl-sn-glycero-3-phosphorylcholine.

In another particular embodiment, the pharmacologically active compound incorporated in the prodrug of the invention is a protein kinase inhibitor. Where the protein kinase inhibitor is a carboxylic acid, the prodrug may be e.g. an ester thereof with a pharmaceutically acceptable alcohol such as glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lysoplasmalogens, lysophospholipids, lyso-phosphatidic acid amides, glycerophosphoric acids, lysophophatidalethanolamine, lyso-phosphatidylethanolamine and N-mono- and N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof. Such a carboxylic acid is e.g. protein kinase inhibitor K252b from Nocardiopsis sp.

Where the protein kinase inhibitor contains an amine group with a replaceable N-linked hydrogen atom, the prodrug may be e.g. an amide thereof with a phosphoric acid derivative selected from glycerophosphoric acids, O-acylated or etherified glycerophosphoric acids, and monoacylated monoetherified glycerophosphoric acids. Such protein inhibitors are e.g. isoquinoline-5-sulfonamide N-substituted by an acyclic or heterocyclic aminoalkyl radical such as $NHCH_2CH_2NHCH_3$ and 2-methylpiperazin-1-yl. Where the protein kinase inhibitor contains at least one phenolic hydroxy group, the prodrug may be

e.g. an ester thereof with a phosphoric acid derivative selected from glycerophosphoric acids, O-acylated glycerophosphoric acids, etherified glycerophosphoric acids, and monoacylated monoetherified glycerophosphoric acids. Such a protein kinase inhibitor is e.g. 4',5,7-trihydroxyisoflavone.

When selecting the intracellular transporting adjuvant for the purposes of the present invention, the skilled person will of course take into consideration the necessity for avoiding such adjuvants, e.g. certain 1,2-diacylglycerols, which are activators of protein kinase C (see Lapetina et al, J. Biol. Chem., 1985, 260: 1358 and Boynton et al, Biochem. Biophys. Res. Comm., 1983, 115: 383), or intracellular transporting adjuvant which are likely to give rise to undesirable products such as these in the cell.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

Example 1: Preparation of Prodrug-1 and Prodrug-2.

Introduction

"Prodrug-1" is the name used herein to denote a 1:1 ester of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) with the choline derivative $ROCH_2$-CH(OH)-CH_2O-(PO_2)-$OCH_2\overset{+}{N}(CH_3)_2$, where R is heptanoyl. BAPTA is a calcium chelator, to which the human cell membrane is normally impermeable, whereas the cell membrane is permeable to prodrug-1, which is not a calcium chelator per se. The carboxylic ester links in prodrug-1 are digestible by PLA_2, so that activated cells such as IgE lymphocytes should exhibit a

selective intracellular accumulation of BAPTA, compared to the unactivated cells, with the result that the $[Ca^{2+}]_i$ level in the activated cells should be reduced when compared with unactivated cells. "Prodrug-2" is the 1:2 ester of BAPTA with the depicted choline derivative.

Procedure

(a) Diheptanoyl-L-α-lecithin

In a dry 3-neck 500 ml flask equipped with oil-sealed stirrer, $CaCl_2$ tube and dropping funnel, were placed 100 ml 5 mm diameter glass beads and 11.0 g (0.01 mole) of $CdCl_2$ adduct of synthetic L-α-glycerophosphoryl-choline. The flask was immersed in an ice-water bath, and to the rapidly-stirred mixture there was added a thin stream of 29.7 g (0.2 mole) freshly prepared heptanoyl chloride dissolved in 60 ml chloroform*, followed by 11 ml (0.14 mole) anhydrous pyridine dissolved in 100 ml chloroform*(*anhydrous, alcohol-free). After 30 minutes, the bath temperature was raised to 25°C and stirring continued for 2 hours. The reaction mixture was poured through a filter-less Buchner, the glass beads washed with 3 x 50 ml chloroform and the combined filtrates clarified by centrifugation. The supernatant was concentrated under reduced pressure, the residue kept for several hours at 0.1 mm vacuum and bath temperature 30-35°C to remove most excess pyridine, and was then stirred with 500 ml anhydrous acetone for 10 minutes, and centrifuged. The precipitate was treated similarly with 2 x 100 ml anhydrous acetone and 2 x 100 ml anhydrous ether. The residual solid material was dried under reduced pressure and freed of the last

traces of cadmium chloride and pyridine hydrochloride, by dissolving in 200 ml of a 5:4:1 by volume mixture of chloroform/methanol/water, and passing the solution through a 120 cm long x 2.5 cm diameter column containing an equivolume mixture of Amberlites IR-45 and IRC-50. The column was washed with 500 ml of the same chloroform/methanol/water mixture, the combined effluents were concentrated to dryness under reduced pressure from a bath at 40-45°C, and the residue dried at 0.1 mm vacuum and 45°C. The crude product was purified by precipitation from a solution in 50 ml chloroform, with 150 ml acetone, centrifugation and recrystallization of the precipitate, 2.3 g (47.6%) from chloroform and ether. (Dioctanoyl-L-α-lecithin can be prepared similarly.)

(b) 1-Heptanoyl-sn-3-glycerophosphorylcholine.

A solution of the product of part (a) (1.2 mmol) in a mixture of ether (196 ml) and methanol (12 ml) was stirred vigorously in presence of $(HOCH_2)_3C\text{-}NH_2.HCl$ (50 ml of 0.1M, pH 8.7) containing $CaCl_2$ (0.72 mM) and 5 mg of crude rattle snake venom (Crotalus adamanteus) as a source of phospholipase A_2, at 37°C for 3 hours. The reaction was monitored by TLC (70:25:4 by volume chloroform/methanol/water). After completion of reaction, the organic layer was separated, and the aqueous layer was washed with ether and then lyophilized. The residue was extracted with 2:1 by volume chloroform/methanol and centrifuged. On evaporation of the clear supernatant, the title product was obtained in 90% yield. Thin layer chromatography using 70:25:4 by volume chloroform/methanol/water showed that it was free from

starting material and heptanoic acid. Any fatty acid in the product can however be remove by crystallization from ethanol-ether. Note: this is a general method for scission of the glycerol-2-ester bond. (Octanoyl-sn-3-glycerophosphoryl-choline can be prepared similarly.)

(c) Prodrug-1 and Prodrug-2

A solution of the product of part (b) (0.5 g, 1.04 mmol) in chloroform (15 ml, freshly distilled over P_2O_5) was added to a solution of BAPTA (0.495 g, 1.03 mmol for the monoester Prodrug-1, or 0.248 g, 0.51 mmol for the diester Prodrug-2), N,N'-dicyclohexyl-carbodiimide (0.214 g, 1.03 mmol) and 4-dimethylaminopyridine (0.025 g, 0.202 mmol) and $HCONMe_2$ (20 ml, freshly distilled over CaH_2) under a nitrogen atmosphere, and the mixture was stirred at room temperature for two days. The reaction was monitored by TLC (65:35:5 by volume chloroform/methanol/water). The precipitate was removed by filtration, the filtrate was concentrated by evaporation in vacuo at 35°C and the residue was dissolved in 2:1:2 by volume chloroform/isopropanol/ water). The organic layer was separated, dried (Na_2SO_4) and then passed through a 20 cm long x 1.8 cm diameter column of silicic acid (Bio-Sil-HA). The column was thoroughly washed with chloroform until free from BAPTA (TLC) and then eluted with a gradient of chloroform/methanol (1:1 by volume) to pure methanol, the elution being monitored by TLC. The eluted fractions were combined and concentrated by evaporation. The desired title product (i.e. Prodrug-1 or Prodrug-2, depending on the number of molar equivalents of BAPTA

used) was crystallized from ether and dried in vacuo over P_2O_5 at 30°C: yield 0.3 g (30%). It will be apparent that the corresponding triester or tetraester may be obtained by varying appropriately number of molar equivalents of BAPTA. (The analogous octanoyl esters are prepared similarly.)

Example 2: Application of Prodrug-1 for reduction of the intracellular calcium level in hyperactivated cells.

Method

Intracellular free $[Ca^{2+}]_i$ content was monitored by flow cytometry using the Ca^{2+}-sensitive dye fluo-3/AM (Molecular Probe Inc., Or.)(see Minta et al, 1989; Kao et al, 1989). Cells obtained from donor blood and those from the blood of an asthmatic patient were further washed twice in DMEM and resuspended to a concentration of 10^7 cells/ml. Fluo-3/AM (1 mM) was prepared in DMSO augmented with the nonionic surfactant Pluronic F-127 (Wyandotte Corp., MI). Aliquots of fluo-3/AM stock solution were added to cell suspensions in DMEM/HEPES at a final concentration of 3 µM (loading buffer). Loading was allowed to proceed for 30 min. at 37°C and continued for 1 hour at 23°C with gentle agitation. Cells were then adjusted to desired concentrations using fresh DMEM/HEPES, supplemented with 2% horse serum. Autofluorescence was eliminated by setting the threshold sensitivity above the levels obtained in absence of dye. Fluorescence intensity data was collected from 5000 single cells and values were expressed as arbitrary fluorescence units. Prodrug-1 (1 mM) was prepared in DMSO and added when appropriate

in final concentration 3 μM to the cells for 5 min. prior to calcium treatment.

Results

Lymphocytes from donor blood and from the blood of an asthmatic patient were exposed to prodrug-1. Accumulation of the liberated BAPTA chelator within the cell was estimated by measurement of $[Ca^{2+}]_i$, by flow cytometry using fluo-3/AM as described above. The results are documented in Figure 1, in which the $[Ca^{2+}]_i$ levels are shown in:

normal lymphocytes (panel A);

normal lymphocytes treated with prodrug-1 (panel B);

lymphocytes from asthmatic patient (panel C);

lymphocytes from asthmatic patient stimulated with IgE (panel D);

lymphocytes from asthmatic patient} (panel C');
treated with prodrug-1 }

and

lymphocytes from asthmatic patient stimulated with IgE} (panel D')
treated with prodrug-1 }.

It is noted that lymphocytes from an asthmatic patient have a double repartition according to the $[Ca^{2+}]_i$ level (panel C). About 50% of the cells exhibit a high $[Ca^{2+}]_i$ level indicating cell hyperactivation, while the second part of the population is similar to the normal one (compare panel A). In the case of panels C' and D', where the cells have been treated with prodrug-1, the population of hyperactivated cells is back to normal, while the population of non-activated cells remains intact (compare panel C). The data demonstrate that prodrug-1 provides selective accumulation of the chelator within activated, but not in non-activated cells.

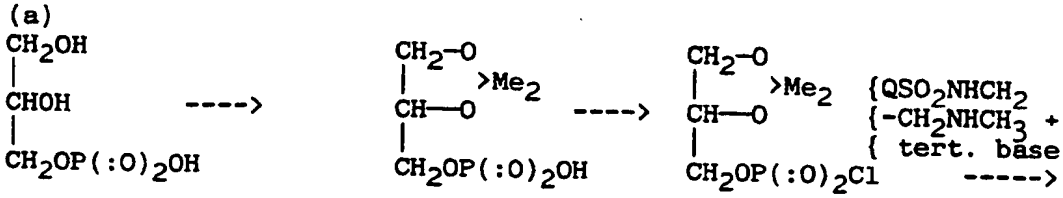
Example 3: Prodrugs of potential application in the treating tumors.

Introduction

In this Example, there are presented a number of illustrative embodiments of the present invention in which a prodrug incorporates a protein kinase inhibitor. After, administration of the prodrug, the inhibitor would be accumulated in a cell exhibiting abnormal proliferation, thus providing potentially an important tool for use in antitumor therapy.

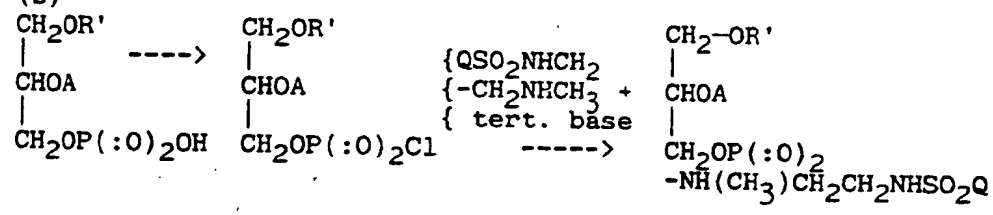
(i) The compound $QSO_2\tilde{N}$ where Q = 5-isoquinolyl and $\tilde{N}$ = $NHCH_2CH_2NHCH_3$, is a selective inhibitor of cAMP-dependent protein kinase: Hidaka et al, Biochemistry, 1984, 23: 5036, and Tash et al, J. Cell Biol., 1986, 103: 649. Similarly, the compound $QSO_2\tilde{N}$ where Q = 5-isoquinolyl and $\tilde{N}$ = 2-methylpiperazin-1-yl, is a potent inhibitor of cyclic nucleotide dependent protein kinase and protein kinase C: Hidaka et al, loc cit, and Kikuchi et al, Nucl. Acid Res., 1988, 16: 10171. These compounds can be covalently conjugated to an intracellular transporting adjuvant by methods known to persons of the art, e.g. illustratively:

(a)



(A)

(b)



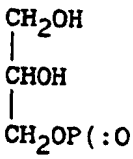
(B)

In scheme (b), R is an aliphatic hydrocarbon group such as is found in plasmalogens (or it may be inserted in a conventional synthetic procedure) and A is an aliphatic acyl radical, e.g. lauroyl, myristoyl, palmitoyl, stearyl and oleyl.

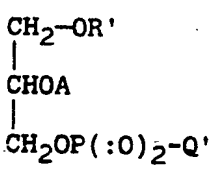
The compound $QSO_2\tilde{N}$ where Q = 5-isoquinolyl and $\tilde{N}$ = 2-methylpiperazin-1-yl, may be attached in a similar manner by means of the piperazine N^4 atom.

It would be expected that the P-N bond in prodrugs (A) and (B) depicted above would be scission-sensitive to enzyme PLD, thus releasing the described protein kinase inhibitors intracellularly, and accumulating these inhibitors in cells having a supranormal level of PLD.

(ii) 4',5,7-trihydroxyflavone is an inhibitor of tyrosine specific protein kinase: Akiyama et al, J. Biol. Chem., 1987, 262: 5592. This compound can be conjugated to an intracellular transporting adjuvant by methods (a) and (b) described in part (i), above. The illustrative conjugates would have structures (C) & (D):



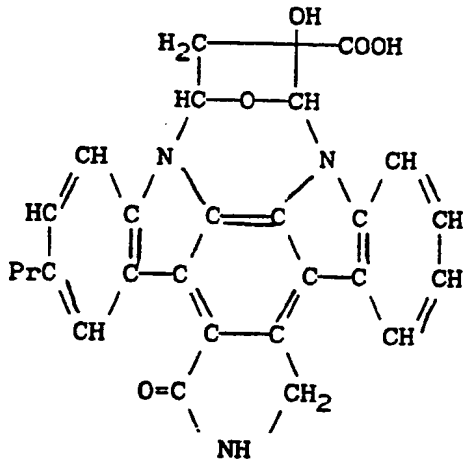
(C)



(D)

where R' and A have the meanings given above and Q' is the residue of 4',5,7-trihydroxyisoflavone from which one phenolic hydrogen atom has been removed and which is thus attached to the rest of the molecule by an O atom forming a P-O bond. It would be expected that this P-O bond in prodrugs (C) and (D) depicted above would be scission-sensitive to enzyme PLD, thus releasing the described protein kinase inhibitors intracellularly, and accumulating these inhibitors in cells having a supranormal level of PLD.

(iii) Protein kinase inhibitor K252b from Nocardiopsis sp. is a carboxylic acid believed to have the following formula:



This compound can be conjugated to an intracellular transporting adjuvant, e.g., by the method described in Example 1, above. Exemplary conjugates are esters of the carboxylic function in the above formula, with e.g. heptanoyl-sn-3-glycerophosphoryl-choline or octanoyl-sn-3-glycerophosphoryl-choline.

Example 4: Preparation and biological properties of DP16.

"DP16" denotes herein to denote a 1:1 ester of BAPTA with the choline derivative $ROCH_2-CH(OH)-CH_2O-OCH_2\overset{+}{N}(CH_3)_2$, where R is hexadecanoyl. DP16 was prepared according to the method described in Example 1.

Introduction to evaluation of DP16 in relation to Ischemia

Bilateral occlusion of the common carotid arteries is the simplest and most direct approach for inducing global ischemia. In the rats there is almost 64% mortality in 24 h later. The causes of mortality are largely brain swelling (edema) and focal lesions (infarcts). Global ischemia is achieved by isolation of the common carotid artery through and incision on the ventral surface of the neck. The salivary glands are moved laterally and the carotid sheath exposed. Both the vagus and sympathetic nerves are separated from the common carotid artery, which is then permanently ligated. Sprague-Dawley rats (250 - 300 g) were anesthetized with halathane or by intramuscular injection of 0.1 ml Ketamine (0.1 g/ml, Park Davis, UK) and 0.1 ml Rompun (2%, Bayer, FRG) per 300 g body weight. DP16 was administered i.p., (0.001 - 0.1 mg/kg) when appropriate following the artery legation. Every experimental and control group included 14 male rats. Statistical analysis was performed according t criteria.

Experimental details and results of Ischemia testing

Embolic stroke: Sprague-Dawley rats (300 g) are anesthetized with halathane. The right common carotid artery is exposed and the

external carotid and pterygopalatine arteries are ligated with No. 0 silk thread. The common carotid artery is cannulated with a plastic tube previously filled with heparinized saline. The canula is then injected (0.5 ml gas-tight Hamilton syringe) with suspension of the spheres, followed by push of 0.5 ml saline. The common carotid artery is then permanently ligated. The polystyrene 15 µm spheres are prepared in 0.05% Tween-80 in normal saline followed by 5 min. of full power sanitation. A 100 µl aliquot is taken and immediately transferred to the syringe.

Ischemia fetal brain model: Sprague-Dawley pregnant rats were used at 20 days gestation. Animals were anesthetized by intramuscular injection of 0.1 ml Ketamine (0.1 g/ml, Park Davis, UK) and 0.1 ml Rompun (2%, Bayer, FRG) per 300 g body weight. An abdominal incision was performed and the two uterine horns were exposed and kept moist throughout the surgery. Intracerebral injection of 1-2mCi/2 ml [^{3}H]arachidonic acid (Na+, 240 mCi/mmol from New England Nuclear, Boston, MA) and/cr 1.5 mCi/2 ml [^{14}C]palmitic acid (Na+, 819 mCi/mmol from Amersham, Searle, UK) in isotonic salt solution containing $NaHCO_3$ (1.32 g%), into the embryos was performed through the uterine wall into the fontanellae. Custom made syringes (33 gauge, 0.375" length from Hamilton, Reno, NV) were used to reduce brain edema. After injection fetuses were returned to the abdominal cavity for maintenance at physiological temperature. After 1h they were subjected to blood flow restriction for 20 min. (restriction session) by clamping the blood vessels in the placenta manifold. Whenever desired, circulation was restored for 30 min. by removal

of the clamps (reperfusion session). At all times both restricted and sham-operated fetuses were maintained in the abdominal cavity before surgical delivery. After delivery through a transverse cut in the uterus, viable fetuses with no apparent edema were killed without delay and excised fetal brains were immediately homogenized in suitable organic solvents for further treatment.

Fetuses cerebral hemispheres model: Fetuses were removed from the uterine horns in a viable state and their cerebral hemispheres were dissected within 15 sec after decapitation. The cerebral hemispheres freed of blood and meninges were separated and each (50±2.5 mg) was placed in a well of a 24-well Falcon culture dish. Tissue was quickly washed twice in cold Dulbecco's Modified Eagle Medium (DMEM, Grand Island Biol. Co) and then incubated at 37°C in 0.6-1.2 ml DMEM flushed with oxygen and supplemented with various additives. Aliquots of incubation medium (0.1 ml) were taken for eicosanoid determination by a radioimmunoasay (RIA) technique. After acidification with 5 ml formic acid, 0.1 ml of isopropanol and 0.5 ml diethylether were added. After mixing and low speed centrifugation (2500 x g, 5 min.) the organic layer was collected and dried under a stream of nitrogen. The resulting residue was dissolved in 0.1 ml sodium phosphate buffer pH 7.4, containing 0.1% bovine serum albumin. Samples were incubated overnight at 4°C with the appropriate polyclonal antiserum, and ^{3}H-labeled tracer (4000 cpm/tube) in a final volume of 0.3 ml. Unbound material was precipitated with 0.3 ml dextran-coated charcoal (Pharmacia, Sweden). After centrifugation at 4°C aliquots of the

supernatant (0.4 ml) were transferred to vials and after addition of scintillation liquid samples were counted in a Packard Tricarb scintillation counter. [^{3}H]Arachidonic acid (240 Ci/mmol) (New England Nuclear, Boston, MA) dissolved in isotonic $NaHCO_3$ (1.32% w/v) was injected through the uterine wall and the fontanellae into the embryonic brain. After injection fetuses were returned to the abdominal cavity for maintenance under physiological conditions. After 1h, fetuses were delivered and immediately sacrificed. Cerebral hemispheres were rapidly excised for subsequent ex vivo incubation or for lipid extraction.

RESULTS. Bilateral Global Cerebral Ischemia causes progressive loss of experimental animals up-to 6-7 days after surgery. As illustrated in Figure 2, DP16 increases post-ischemic recovery by 250%, compared with control using non-protected rats ($p < 0.01$). This data demonstrates the potential ability of DP 16 to treat otherwise fatal ischemic conditions.

Heart Ischemia - perfused heart model: White rats were sacrificed by cervical dislocation and their hearts were rapidly removed and reperfused at 60 mmHg with modified Krebs-Henselleit buffer utilizing a Langendorff perfused heart model. Hearts were perfused for 10-min. preequlibration interval and were subsequently rendered either global ischemic (zero flow) or continuously perfused for the indicated time. Perfusion were terminated by rapid excision of ventricular tissue and directly submersion into cold homogenization buffer (10 mM imidazole, 10 mM KCl, .25 M sucrose [grade 1], pH 7.8) Both the activation of phospholipase A2 and its reversibility during reperfusion were

temporally correlated to alterations in myocytic anaerobic metabolism and electron microscopic analyses.

Model of ventricular fibrillations causing by coronary occlusion: Dogs (11.6 - 20.7 kg) were anesthetized and instrumented to measure left circumflex coronary blood flow, left ventricular pressure, and ventricular electrogram. The left anterior descending artery was ligated and an anterior wall myocardial infarction was then produced. All leads to the cardiovascular instrumentation were tunneled under the skin to exit on the back of the animal's neck. Appropriate medicine was given to minimize postoperative pain and prevent inflammation. The ischemia test was performed after 3-4 weeks.

Properties of DP 16 in relation to the treatment of epileptic disorders

Pilocarpine based model of experimental epilepsy: Acetylcholine, acetylcholinesterase inhibitors and acetylcholine analogues are effective epileptogenic agents when applied intracerebrally or systematically (see ref. in Leite et al., Neurosci. & Biobeh. Rev., 1990, 14:511- 17). It was demonstrated in different species that systemic administration of muscarinic cholinergic agonists produced electroencephalographic and behavioral limbic seizure accompanied by widespread brain damage resembling topographically that produced by kainic acid and folates and are frequently observed in autopsied human epileptics. Systemic injections of the pilocarpine, a potent muscarinic cholinergic agonist, are capable of producing a sequence of behavioral alterations including stirring spells, facial automatisms and motor limbic

seizures, that develop over 1 - 2 hours and build progressively into limbic status and following by general status epilepticus.

RESULTS. Immediately following injection of pilocarpine, akinesia, ataxic lurching, facial automatism and heart tremor dominated the animals' behavior. Further development of epileptic events is dose - dependent (Figure 3). Administration of pilocarpine in doses of 300 - 350 mg/kg causes appearance of limbic seizures with rearing, forelimb clonus, salivation, intense masticatory jaw movements and falling. Motor limbic seizures commenced after 20 - 30 min., recurred every 2 - 8 min and lead to status epilepticus. Increase of the dose of pilocarpine up-to 400mg/kg abolished limbic seizures and after 15 - 25 min of initial behavioral alterations causes fatal general tonic - clonic convulsions. We consider this dose as LD_{100}.

Administration of DP16 prior to pilocarpine prevented death in the animals and decreased epileptiform manifestations. As shown in Figure 4, DP16 exhibits a therapeutic at doses in the range 10^{-8} to 10^{-5} mg/kg. For this particular model of epilepsy (pilocarpine 400 mg/kg; rats) the estimated therapeutic index (ET) of DP16 is 0.5 mg/kg/$5x10^{-7}$ mg/kg = $1x10^{6}$. The data obtained suggest that DP16 is an extremely promising prodrug for the treatment of epileptic disorders.

Pilocarpine and cardiotoxicity.

Two types of death were found in rats treated with pilocarpine, firstly due to fatal convulsions and secondly, retarded death not immediately due to epileptic events. We attempted to understand the actual reason of retarded death of

rats after pilocarpine-induced convulsions. Under macroscopic autopsy of these animals were seen signs of cardiopulmonary damages: lung edema and hemorrhages, dilated and in same cases deformed hearts. Dyeing of hearts with 0.1% Trypan blue in surviving animals revealed spotted picture of myocardia with areas of intensive dye absorption, i.e., damaged parts, and pale areas, i.e., infarctions. Thus, we can consider that after pilocarpine administration, there developed heart damage, which we term post-pilocarpine-seizure-cardiopathy (PSCP). Studies of PSCP in relation to DP16 evaluation were performed in vivo and in vitro with rats which survived after convulsive and sub convulsive doses of Pilocarpine.

PSCP Experiments: Adult (2-3 months) male Sprague-Dawley rats were used for all experiments. They were fed with standard briquette chow with water ad libitum and were maintained in standard plastic cages (4-5 individuals in each cage) under natural illumination. A pilocarpine-scopolamine epileptic status model (pilocarpine) was performed as described earlier. In a group of 23 rats, pilocarpine was administered i.p. in different doses which ranged from 100 to 400 mg/kg body weight (B/W) for different periods of time; a second group of 17 rats was treated with DP16 prior to pilocarpine administration, wherein the DP16 was injected for 30 min before pilocarpine in the next dose range and its effect was investigated in the ensuing periods.

In vivo ECG (Birtcher-Cardio-Tracer, model 375, USA) in three standard leads were recorded under ketamine anesthesia (3.3 mg/kg Imalgene 100, Rhone Merieux, France and 7 mg/kg Rompun, Bayer Leverkusen, Germany, i.m.). ECG recordings were made in the period before pilocarpine injections (control), 24h after pilocarpine administration (acute period) and after relative stabilization of cardiac function, on the 3-14th day after pilocarpine administration. Part of the ECG recordings were made under nembutal anesthesia (35 mg/kg, i.p.) in the period before establishing Langendorff's perfusion isolated heart preparation. Perfusion-Hypoxia-Reperfusion isolated heart model (PHR) was performed with the conventional Langendorff technique (non-recirculating perfusion system) adjusted to 37°C in two modifications: 1. under constant Perfusion Pressure (PP)--60 mm Hg; or 2. under constant flow, established after the first 10-15 min perfusion with PP as above, by adjusting flow with help of peristaltic pump (Ismatec SA, Laboratoriumstechnic, Switzerland). In the case of constant PP the volume of effluent flow was measured on electron balance (Precisa 1000C-3000D, Switzerland). In case of constant flow, established at the control period, flow did not change during subsequent experimental periods and PP was recorded frequently. After 30 min of the control period, perfusion was stopped for 30 min and subsequent reperfusion period lasted 30 min. Direct ECG were recorded from ventricular apex (lead 1), auriculum (lead 2) and in-between (lead 3). The coronary vessel's perfusion resistance (CVPR) was calculated in arbitrary units as follows: PP/flow/heart weight. Following the

protocol above, hearts were subjected to perfusion with the dye Trypan blue (0.1%), in order to evaluate cellular damage and infarction.

RESULTS AND DISCUSSION

ECG results in vivo are illustrated in Figure 5, in which open bars reflect some ECG events, expressed as mean ±SE from individual ECGs in control period. The first group of bars demonstrates ECG changes after pilocarpine injections in an acute stage of PSCP: statistically significant depressions of R- peak are noted under leads 1 and 2 (47% & 16% of control one respectively). DP16 treatment of PSCP normalized electrical activity at the acute stage in 5 out of 7 treated rats. It is known that the amplitude of ECG events are partly connected with the intensity of correspondent physiological processes. Thus, the pilocarpine-induced change of R-wave and its normalization by DP16 may reflect the ability of DP16 to cure ventricular weakness, at least under PSCP. Control rats display relative normalization of R-wave in 3 - 14 days after pilocarpine. However, R normalization somehow correlated with drastically increased S-wave depth under lead 3 (36%) and lead 2 (61%).(the last is not yet statistically significant in view of large variability.) Increase of S-wave depth reflected damage of myocardial ischemia & possibly suggesting infarction in Pilocarpine treated control animals. As during the acute stage of PSCP in the phase of stabilization, DP 16 prevents the appearance of ECG alterations noted in control rats. The difference between animals protected with DP16 and those not

protected, is statistically significant ($p<0.01$). In this period PSCP there is marked elevation of Heart Rate as in control Pilocarpine, as in DP16 treated animals. Such tachycardia possibly connected with hemodynamic insufficiency, which is characteristic for infarction pathophysiology. Thus, in vivo ECG investigation during long-term period after Pilocarpine injections revealed definite alteration of cardiac functions (PSCP), which in some animals may be cured by DP16-treatment.

Langendorff's Heart Model. Figure 6 shows Coronary Vessels Perfusion Resistance (CVPR) in isolated Langendorff's hearts. In the first 30 min of control isolated Langendorff's hearts CVPR steadily increased and this elevation is statistically significant after 20 min. In all hearts, perfused after pilocarpine administrations, initial perfusion flow was larger then in control, and subsequent CVPR significantly decreased (bottom line). This decrease of coronary vessels' tone possibly connected with intracardial noradrenaline deficiency or paralysis, evoked by hypoxia. Treatment of rats with DP 16 prior to pilocarpine application prevents damage of CVPR regulation in both the initial and final periods of perfusion, thus providing evidence relating to the ability of DP 16 to normalize coronary vessels function under hypoxic conditions. Cessation of perfusion for 30 min and subsequent reperfusion is characterized by the well-known broad class of cardiac damage events, which we classified with an arbitrary scale as shown in Figure 7. Control hearts from non-treated rats mostly restored after stopping of perfusion with distinct range of alterations (as impaired

myocardial excitability, conductivity and contractility). Mean point of recovery in control group is 6.3±0.6 (n=7). Hearts from pilocarpine-treated rats on different stages of PSCP demonstrated an increase of the spectrum and severity of pathological events, as the mean point of recovery was just 3.3±0.8, n=7, $p<0.05$. Recovery was frequently accompanied by ventricular fibrillation. Some of the hearts were not restored completely or restored atrial activity only. DP 16 treatment prior to pilocarpine administrations increased ability of damaged hearts to restore after reperfusion cessation: the mean point was 6.4±0.6 (n=9). In this group of rats we met more often with cases of complete recovery. Thus, DP16 treatment of pilocarpine-induced heart damage (PSCP) produced a definite improvement in cardiac function.

Investigation of antiepileptic effects of DP16: Metrazol minimal seizures test.

Method

Trial of DP16 as a possible antiepileptic drug was performed on 3-4 week old male BALB/c mice (18 - 27 g). Animals were maintained on an adequate diet and allowed free access to food and water except briefly during the experimental period. Animals were separately housed for one hour in transparent plastic cages before treatment and during the experimental period. Drugs were dissolved in normal saline with injection volume adjusted to 0.01 ml/g of body weight. DP16 was administered i.p., in doses ranging from 0.1 to 300 µg/kg: (0.1

µg/kg: n=10, 5 µg/kg: n=10, 25 µg/kg: n=20, 75 µg/kg: n=20, 150 µg/kg: n=20, and 300 µg/kg: n=10 animals respectively). Control animals received injections i.p. of normal saline. DP16 or saline administration followed in 30 minutes by Metrazol (50 µg/kg, s.c.). Subsequently epileptic signs were observed for the next 30 minutes. Absence or relative delay of myoclonic jerks (MJ) in the experimental group was considered as indication of possible antiepileptic activity. Data were analyzed according to method c2 (chi-square) with the computer statistic package "StatViewII".

Results and Conclusions

Metrazol in a dosage of 50 µg/kg, s.c. caused myoclonic jerks (MJ) in all of control mice with a latent period of 1011 min (n=11). The effect of DP16 on the appearance of minimal metrazol induced seizures is shown in Figure 8. Mice treated with 0.1 µg/kg DP16 showed the same response to metrazol as control (untreated) animals. DP16 in doses ranging from 5 to 300 µg/kg exhibited a significant protective effect ($p < 0.001$). The results of the test suggest a significant dose-dependent antiepileptic effect of DP16 on the metrazol induced seizures.

Investigation of cardioprotective effect of DP16:

1. Trial on ex vivo rat heart Low-flow - Reperfusion model.

Method and Results

Broadly used ex vivo Langendorff's heart Stop-flow - Reperfusion and Low-flow models (Neely and Rovetto, 1975) remained conventional for pharmacological trials. Cardioprotective effect of DP16 was evaluated in a combined

experimental paradigm of Normal flow perfusion followed by Low-flow and then by Reperfusion (LFR) of ex vivo rat heart. Evolution of ECG and of perfusion pressure (PP) was considered as a criterion for the drug evaluation. Data collected in experiments in the presence of DP16 were compared with one without drug supplement (control no. 1). An additional set of experiments (control no. 2) was performed with mixture of the components comprising DP16: BAPTA & lysophosphatidylcholine (LPC). DP16 (1 - 100 µg/L) was dissolved in a regular perfusion buffer. The mixture of BAPTA & LPC was dissolved in DMSO as a stock solution; the final concentration of BAPTA, LPC and DMSO in the perfusion buffer for control no. 2 was 100 µg/L of each component.

A severe decrease in perfusion pressure (PP) below 20 mm Hg (low-flow period) caused a sinus bradycardia culminated by stable AV block (Fig. 9.2, c.f. normal flow in Fig. 9.1) frequently with ventricular arrhythmia (9 experiments, control 1). Long term (> 0.5h) low-flow conditions usually ended by paroxysmal tachyarrhythmia and ventricular fibrillation (VF). Reperfusion started before VF could temporary restore sinus rhythm. However, reperfusion in most of the control experiments caused increase of coronary vascular tone and arrhythmia followed by irreversible VF (Fig. 9.3). After establishment of AV block in low-flow period perfusion medium was supplemented by the drugs. No therapeutic effect on AVB was observed in the experiments with mixture of BAPTA & LPC (Fig. 10.3 and 10.4, c.f. without BAPTA and LPC - normal flow in Fig. 10.1 and low flow

perfusion in Fig. 10.2). While addition of DP16 caused complete or temporal relief of atreoventricular synchronism (4 and 1 cases respectively) (Fig. 11.3). Moreover, DP16 exhibited notable cardioprotective effect in the reperfusion period: full restoration of the sinus rhythm was observed in 4 out of 5 experiments (Fig. 11.4; c.f. without DP16 - normal flow in Fig. 11.1 and low flow perfusion in Fig. 11.2). ECG analysis revealed mainly metabolic type of DP action: enhancing of atrial (definite increase of the heart rate) and ventricular (restoration of regular sinus rhythm) excitability. However, residual delay of AV conductivity (increased PQ interval) was observed.

Conclusions

The data obtained in this experiment suggest significant cardioprotective activity of DP16 in ischemia - reperfusion pathology.

Investigation of cardioprotective effect of DP16:
2. Trial on in vivo model of myocardial damage.

Method and Results

Administration of the potent β-adrenoreceptor agonist isoproterenol (ISO) is commonly accepted model of experimental myocardial pathology. The cardioprotective effect of DP16 was tested on 82 Sprague-Dawley female rats weighing 250-350 g. Myocardial damage was induced in rats by two consecutive injections of ISO (85 μg/kg, s.c.). When appropriate, the injections of ISO were followed in 30 and 180 minutes by DP16 (0.01 μg/kg, i.p.). The effect of DP16 was estimated by ECG

analysis and determination of serum glutamic-oxaloacetat transaminase (SGOT) and lactatdehydrogenase (LDH) activity. Mortality of control rats after ISO intervention was 17.1±5.9% (7 out of 41). The surviving animals exhibited striking hyperacute deviation ST-segment in lead 1 and 2 ECG. Pathological signs on ECG were aggravated during the experimental period. In 48 hours after the second ISO injection all treated animals displayed pathological displacement of ST-segment. Administration of DP16 decreased mortality in 2 cases (2 out of 30). Animals receiving DP16 exhibited significantly ($p < 0.05$) fewer alterations in the ECG. Pathological displacement of the ST-segment was found only on 28 and 40% of ECG (in 24 and in 48 hours following ISO respectively). Biochemical determination demonstrated a 1.7 - 1.9 fold increase if SGOT and LDH in ISO treated control rats ($p<0.05$). Treatment with DP16 substantially decreased the percentage of experimental animals exhibiting abnormal level of SGOT and LDH activity.

Conclusions

The data above suggest a significant cardioprotective effect of DP16 in an in vivo model of myocardial pathology.

GENERAL CONCLUSIONS. The prodrug denoted DP16 exhibited significant therapeutic and protective effects in experimental models of stroke & ischemia as well as in models of epilepsy, comparable with using the corresponding drug in conventional form in an amount which is 10^5 - 10^6 times the amount when used in the form of the prodrug of the invention.

Example 5: Preparation of Prodrug-3.

"Prodrug-3" is the name used herein to denote a 1:1 ester of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) with 1-myristylmyristyl alcohol and is prepared as follows. A solution of BAPTA (0.5 g, 1.05 mmol) in dimethylformamide (25 ml, freshly distilled over CaH_2), 1-myristylmyristyl alcohol (0.451 g, 1.1 mmol), N,N'-dicyclohexylcarbodiimide (0.216 g, 1.1 mmol) and 4-dimethylaminopyridine (0.025 g, 0.202 mmol) were stirred together for two days at room temperature under argon, in a 50 ml flask equipped with a magnetic stirrer. After two hours, N,N'-dicyclohexylurea began to precipitate. The reaction was monitored by TLC (90:10 v/v chloroform:methanol); R_f of the product = 0.62. The precipitate was removed by filtration and the filtrate was concentrated at 35°C in vacuum. The residue was extracted with 25 ml of a 2:1:2 v/v mixture of chloroform:isopropanol:water. The organic layer was separated, washed with 1% aq. NaCl solution and dried over Na_2SO_4; it was then evaporated and the residue was passed through a 160x30 mm column of Kieselgel 60 (230-400 mesh ASTM), the desired product being eluted with a 90:10 v/v chloroform:methanol mixture. The 1-myristylmyristyl alcohol was prepared according to the method of Molotkovski, V.G. and Bergelson, L.D. (Biologicheska Chimia, 1982, 8(9): 1256-1262). The BAPTA-1-myristylmyristyl alcohol ester link in Prodrug-3 is susceptible to digestion by esterases.

Example 4: Preparation and biological properties of TVA16.

"TVA16" is the name used herein to denote a 1:1 ester of valproic acid with the choline derivative $ROCH_2-CH(OH)-CH_2O-OCH_2\overset{+}{N}(CH_3)_2$, where R is hexadecanoyl, and was prepared as follows. A solution of 1-hexadecanoyl-sn-glycero-3-phosphorylcholine (1.04 mmol) in chloroform (25 ml, freshly distilled over P_2O_5), valproic acid (0.159 g, 1.1 mmol), N,N'-dicyclohexylcarbodiimide (0.216 g, 1.1 mmol) and 4-dimethylaminopyridine (0.025 g, 0.202 mmol) were stirred together for two days at room temperature under argon, in a 50 ml flask equipped with a magnetic stirrer and glass beads (10 g, 5 mm diameter). After two hours, N,N'-dicyclohexylurea began to precipitate. The reaction was monitored by TLC (65:25:4 v/v chloroform:methanol:water); R_f of the product = 0.41. The precipitate and glass beads were removed by filtration and the filtrate was concentrated at 35°C in vacuum. The residue was extracted with 25 ml of a 2:1:2 v/v mixture of chloroform:isopropanol:water. The organic layer was separated, washed with 1% aq. NaCl solution and dried over Na_2SO_4; it was then evaporated and the residue was passed through a 160x30 mm column of Kieselgel 60 (230-400 mesh ASTM), the desired product being eluted with a 65:25:4 v/v chloroform:methanol:water mixture; R_f=0.4.

A test sample of TVA16 was administered i.p. (0.01 to 100 mg/kg) to a group of three mice, one hour before an s.c. dose of metrazol (80 mg/kg). An effective dose was the amount which prevented convulsions (scored 2 points per animal) and/or death

(scored 1 point per animal) in the subsequent 30 minutes. On this basis, the ED_{100} could be calculated and is compared to known anticonvulsants in the following table.

Anticonvulsant activity (ED_{100}, mg/kg) of known drugs and TVA16

chlordiazepoxide	25	muscimol (i.p.)	2.5
diazepam	2.5	nifedipine	>100
diphenylhydantoin	>100	nimodipine	>300
flunarizine	>300	phenobarbital	50
glutethimide	150	sodium valproate	500
meprobamate	200	verapamil	>100
MK-801	0.5	TVA16	0.6

From the above data it may be seen that TVA16 has significant anticonvulsant activity and appears to be more than 500x as potent as sodium valproate.

While the present invention has been particularly described, persons skilled in the art will appreciate that many variations and modifications can be made. Therefore, the invention is not to be construed as restricted to the particularly described embodiments, rather the scope, spirit and concept of the invention will be more readily understood by reference to the claims which follow.

CLAIMS

1. A pharmaceutically acceptable prodrug which is a covalent conjugate of a pharmacologically active compound and an intracellular transporting adjuvant, characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity.

2. A prodrug according to claim 1, wherein said pharmacologically active compound is a pharmacologically active carboxylic acid and said adjuvant comprises at least one pharmaceutically acceptable alcohol which is selected from glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lyso-plasmalogens, lysophospho-lipids, lysophosphatidic acid amides, glycerophosphoric acids, lyso-phophatidalethanolamine, lysophosphatidyl-ethanolamine and N-mono- & N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof.

3. A prodrug according to claim 2, wherein said pharmacologically active carboxylic acid is selected from branched-chain aliphatic carboxylic acids, salicylic acids, steroidal carboxylic acids, monoheterocyclic carbocylic acids and polyheterocyclic carboxylic acids.

4. A prodrug according to claim 1, which is a partially or totally esterified carboxylic acid (a) with hydroxy compound (b), where (a) is a pharmaceutically acceptable chelating agent for

calcium having the formula $(HOOC-CH_2-)_2-N-A-N-(-CH_2COOH)_2$ where A is saturated or unsaturated, aliphatic, aromatic or heterocyclic linking radical containing, in a direct chain link between the two depicted nitrogen atoms, 2-8 carbon atoms in a continuous chain which may be interrupted by 2-4 oxygen atoms, provided that the chain members directly connected to the two depicted nitrogen atoms are not oxygen atoms, and (b) is a pharmaceutically acceptable alcohol containing 3 to 32 carbon atoms and 1-3 hydroxyl radicals; and salts with alkali metals of said partially esterified carboxylic acids, as well as acid addition salts of such of said esterified carboxylic acids as contain one or more potentially salt-forming nitrogen atoms.

5. A prodrug according to claim 4, wherein said pharmaceutically acceptable alcohol is a C_{7-32} secondary monohydric alcohol.

6. A prodrug according to claim 4, wherein said pharmaceutically acceptable alcohol contains 3 to 6 carbon atoms and 1-3 hydroxyl radicals.

7. An ester according to claim 4, wherein said linking radical A is a member selected from the group consisting of $-(CH_2CH_2)_m-$ where m = 1-4, in which 2-4 of the carbon atoms not attached to nitrogen may be replaced by oxygen atoms, and $-CR=CR-O-CH_2CH_2-O-CR'=CR'-$, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, complete an aromatic or heterocyclic ring containing 5 or 6 ring atoms, the

ring completed by R-R being the same as or different from the ring completed by R'-R'.

8. An ester according to claim 4, wherein said linking radical A is selected from the group consisting of $-CH_2CH_2-$ and $-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-$.

9. An ester according to claim 4, wherein said linking radical is $-CR=CR-O-CH_2CH_2-O-CR'=CR'-$, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, complete an aromatic or heterocyclic ring which is selected from the group consisting of furan, thiophene, pyrrole, pyrazole, imidazole, 1,2,3-triazole, oxazole, isoxazole, 1,2,3-oxadiazole, 1,2,5-oxadiazole, thiazole, isothiazole, 1,2,3-thiadiazole, 1,2,5-thiadiazole, benzene, pyridine, pyridazine, pyrimidine, pyrazine, 1,2,3-triazine, 1,2,4-triazine, and 1,2-, 1,3- and 1,4-oxazines and -thiazines, the ring completed by R-R being the same as or different from the ring completed by R'-R'.

10. An ester according to claim 9, wherein the linking radical A is $-CR=CR-O-CH_2CH_2-O-CR'=CR'-$, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, completes the same or different rings selected from unsubstituted and substituted benzene rings, in which substituted benzene rings contain 1-4 substituents selected from the group consisting of C_{1-3}-alkyl, C_{1-3}-alkoxy, fluorine, chlorine, bromine, iodine and CF_3, or a single divalent substituent which

is $-O-(CH_2)_n-O-$ and n = 1-3.

11. An ester according to claim 4, wherein said chelating agent is selected from ethylene-1,2-diamine-N,N,N',N'-tetraacetic acid, ethylene-1,2-diol-bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid and 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

12. An ester according to any of claims 7 to 11, wherein said pharmaceutically acceptable alcohol contains 3 to 6 carbon atoms and 1-3 hydroxyl radicals.

13. An ester according to claim 12, wherein said pharmaceutically acceptable alcohol comprises at least one member of the group consisting of glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lyso-plasmalogens, lysophospholipids, lysophosphatidic acid amides, glycerophosphoric acids, lysophophatidalethanolamine, lyso-phosphatidylethanolamine and N-mono- and N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof.

14. An ester according to claim 4, which is selected from the mono-, di-, tri- and tetra- esters of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid with heptanoyl-sn-3-glycerophosphoryl-choline or octanoyl-sn-3-glycerophosphoryl-choline.

15. A prodrug according to claim 1, wherein the pharmacologically active compound is a protein kinase inhibitor.

16. A prodrug according to claim 15, which is an ester of a protein kinase inhibitor carboxylic acid with a pharmaceutically acceptable alcohol comprising at least one member of the group consisting of glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lyso-phosphatidic acids, lysoplasmalogens, lysophospholipids, lyso-phosphatidic acid amides, glycerophosphoric acids, lysophophatidal-ethanolamine, lysophosphatidyl-ethanolamine and N-mono- and N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof.

17. A prodrug according to claim 16, wherein the protein kinase inhibitor is protein kinase inhibitor K252b from Nocardiopsis sp.

18. A prodrug according to claim 15, wherein the protein kinase inhibitor contains an amine group with a replaceable N-linked hydrogen atom, and the prodrug is an amide thereof with a phosphoric acid derivative selected from the group consisting of glycerophosphoric acids, O-acylglycerophosphoric acids, etherified glycerophosphoric acids, and monoacylated monoetherified glycerophosphoric acids.

19. A prodrug according to claim 18, wherein the protein kinase inhibitor is isoquinoline-5-sulfonamide which is N-substituted by an acyclic or heterocyclic aminoalkyl radical.

20. A prodrug according to claim 19, wherein said aminoalkyl radical is selected from the group consisting of $NHCH_2CH_2NHCH_3$ and 2-methylpiperazin-1-yl.

21. A prodrug according to claim 15, wherein the protein kinase inhibitor contains at least one phenolic hydroxy group, and the prodrug is an ester thereof with a phosphoric acid derivative selected from the group consisting of glycerophosphoric acids, O-acyl- glycerophosphoric acids, etherified glycerophosphoric acids, and monoacylated monoetherified glycerophosphoric acids.

22. A prodrug according to claim 21, wherein the protein kinase inhibitor is 4',5,7-trihydroxyisoflavone.

23. A technique for treating a condition or disease in a human related to supranormal intracellular enzyme activity, which comprises administering to a human having such condition or disease, in an amount effective for reducing the supranormal enzyme activity, a pharmaceutically acceptable cell membrane permeable prodrug, said prodrug being a covalent conjugate of a cell membrane impermeable pharmacologically active compound and an intracellular transporting adjuvant, characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity, such that the bond is broken in response to such activity, whereby the pharmacologically active compound accumulates selectively within cells having supranormal intracellular enzyme activity.

24. A technique according to claim 23, wherein said pharmacologically active compound is a pharmacologically active carboxylic acid and said adjuvant comprises at least one pharmaceutically acceptable alcohol which is selected from glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lyso-plasmalogens, lysophospholipids, lysophosphatidic acid amides, glycerophosphoric acids, lysophophatidal-thanolamine, lysophosphatidyl-ethanolamine and N-mono- and N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof.

25. A technique according to claim 24, wherein said pharmacologically active carboxylic acid is selected from branched-chain aliphatic carboxylic acids, salicylic acids, steroidal carboxylic acids, monoheterocyclic carbocylic acids and polyheterocyclic carboxylic acids.

26. A technique according to claim 23, wherein said supranormal enzyme activity is in turn related to an excess of intracellular Ca^{2+} ions, and said pharmacologically active compound is a calcium chelating agent.

27. A technique according to claim 26, wherein said prodrug is a partial or total ester of chelating agent (a) with alcohol (b), where (a) is a pharmaceutically acceptable chelating agent for calcium having the formula $(HOOC\text{-}CH_2\text{-})_2\text{-N-A-N-}(\text{-}CH_2COOH)_2$ where A is saturated or unsaturated, aliphatic, aromatic or

heterocyclic linking radical containing, in a direct chain link between the two depicted nitrogen atoms, 2-8 carbon atoms in a continuous chain which may be interrupted by 2-4 oxygen atoms, provided that the chain members directly connected to the two depicted nitrogen atoms are not oxygen atoms, and (b) is a pharmaceutically acceptable alcohol containing 3 to 32 carbon atoms and 1-3 hydroxyl radicals; and salts with alkali metals of said partially esterified carboxylic acids, as well as acid addition salts of such of said esterified carboxylic acids as contain one or more potentially salt-forming nitrogen atoms.

28. A technique according to claim 27, wherein said pharmaceutically acceptable alcohol is a C_{7-32} secondary monohydric alcohol.

29. A technique according to claim 27, wherein said pharmaceutically acceptable alcohol contains 3 to 6 carbon atoms and 1-3 hydroxyl radicals.

30. A technique according to claim 27, wherein said linking radical A is a member selected from the group consisting of $-(CH_2CH_2)_m-$ where m = 1-4, in which 2-4 of the carbon atoms not attached to nitrogen may be replaced by oxygen atoms, and -CR=CR-O-CH_2CH_2-O-CR'=CR'-, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, complete an aromatic or heterocyclic ring containing 5 or 6 ring atoms, the ring completed by R-R being the same as or different from the ring completed by R'-R'.

31. A technique according to claim 27, wherein said linking radical A is selected from the group consisting of $-CH_2CH_2-$ and $-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-$.

32. A technique according to claim 27, wherein said linking radical is $-CR=CR-O-CH_2CH_2-O-CR'=CR'-$, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, complete an aromatic or heterocyclic ring which is selected from the group consisting of furan, thiophene, pyrrole, pyrazole, imidazole, 1,2,3-triazole, oxazole, isoxazole, 1,2,3-oxadiazole, 1,2,5-oxadiazole, thiazole, isothiazole, 1,2,3-thiadiazole, 1,2,5-thiadiazole, benzene, pyridine, pyridazine, pyrimidine, pyrazine, 1,2,3-triazine, 1,2,4-triazine, and 1,2-, 1,3- and 1,4-oxazines and -thiazines, the ring completed by R-R being the same as or different from the ring completed by R'-R'.

33. A technique according to claim 32, wherein the linking radical A is $-CR=CR-O-CH_2CH_2-O-CR'=CR'-$, where each of the pairs of radicals R-R and R'-R', together with the attached -C=C- moiety, completes the same or different rings selected from unsubstituted and substituted benzene rings, in which substituted benzene rings contain 1-4 substituents selected from the group consisting of C_{1-3}-alkyl, C_{1-3}-alkoxy, fluorine, chlorine, bromine, iodine and CF_3, or a single divalent substituent which is $-O-(CH_2)_n-O-$ and n = 1-3.

34. A technique according to claim 27, wherein said chelating agent is selected from ethylene-1,2-diamine-N,N,N',N'-tetraacetic acid, ethylene-1,2-diol-bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid and 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

35. A technique according to any of claims 30 to 34, wherein said pharmaceutically acceptable alcohol contains 3 to 6 carbon atoms and 1-3 hydroxyl radicals.

36. A technique according to claim 35, wherein said pharmaceutically acceptable alcohol comprises at least one member of the group consisting of glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lyso-plasmalogens, lysophospholipids, lysophosphatidic acid amides, glycerophosphoric acids, lysophophatidalethanolamine, lysophosphatidyl-ethanolamine and N-mono- and N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof.

37. A technique according to claim 27, wherein said ester is selected from the mono-, di-, tri- and tetra- esters of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid with heptanoyl-sn-3-glycerophosphoryl-choline or octanoyl-sn-3-glycerophosphoryl-choline.

38. A technique according to claim 23, wherein said pharmacologically active compound is a protein kinase inhibitor.

39. A technique according to claim 38, wherein said prodrug is an ester of a protein kinase inhibitor carboxylic acid with a pharmaceutically acceptable alcohol comprising at least one member of the group consisting of glycerol, C_{3-20} fatty acid monoglycerides, C_{3-20} fatty acid diglycerides, hydroxy-C_{2-6}-alkyl esters of C_{3-20} fatty acids, hydroxy-C_{2-6}-alkyl esters of lysophosphatidic acids, lysoplasmalogens, lysophospholipids, lysophosphatidic acid amides, glycerophosphoric acids, lysophophatidal-ethanolamine, lysophosphatidyl-ethanolamine and N-mono- and N,N-di-(C_{1-4})-alkyl and quaternated derivatives of the amines thereof.

40. A technique according to claim 39, wherein the protein kinase inhibitor is protein kinase inhibitor K252b from Nocardiopsis sp.

41. A technique according to claim 38, wherein the protein kinase inhibitor contains an amine group with a replaceable N-linked hydrogen atom, and the prodrug is an amide thereof with a phosphoric acid derivative selected from the group consisting of glycerophosphoric acids, O-acylglycerophosphoric acids, etherified glycerophosphoric acids, and monoacylated monoetherified glycerophosphoric acids.

42. A technique according to claim 38, wherein the protein kinase inhibitor is isoquinoline-5-sulfonamide which is N-substituted by an acyclic or heterocyclic aminoalkyl radical.

43. A technique according to claim 42, wherein said aminoalkyl radical is selected from the group consisting of $NHCH_2CH_2NHCH_3$ and 2-methylpiperazin-1-yl.

44. A technique according to claim 38, wherein the protein kinase inhibitor contains at least one phenolic hydroxy group, and the prodrug is an ester thereof with a phosphoric acid derivative selected from the group consisting of glycerophosphoric acids, O-acyl-glycerophosphoric acids, etherified glycerophosphoric acids, and monoacylated monoetherified glycerophosphoric acids.

45. A technique according to claim 44, wherein the protein kinase inhibitor is 4',5,7-trihydroxyisoflavone.

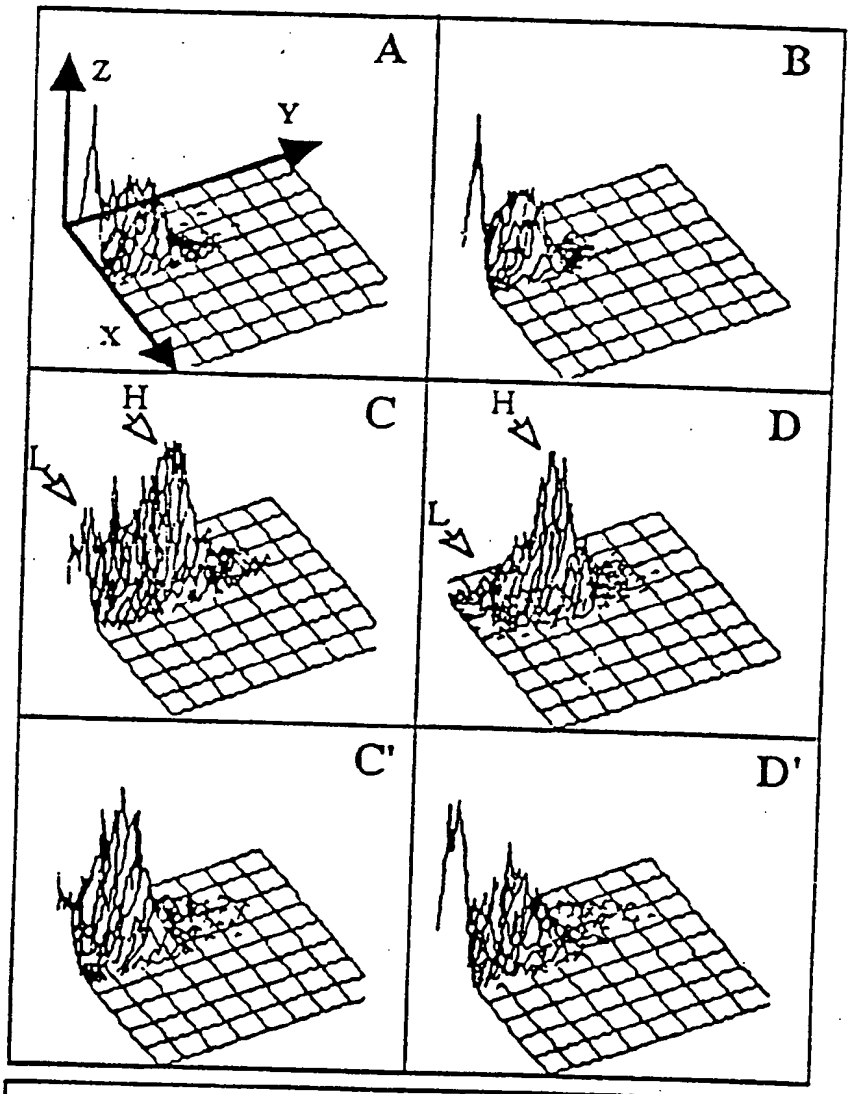
46. Use for the manufacture of a medicament for treating a condition or disease in a human related to supranormal intracellular enzyme activity, by selectively accumulating a cell membrane impermeable pharmacologically active compound within cells having such activity, of a pharmaceutically acceptable cell membrane permeable prodrug, said prodrug being a covalent conjugate of said pharmacologically active compound and an intracellular transporting adjuvant, characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity, such that the bond is broken in response to such activity.

47. Use according to claim 46, wherein said prodrug is as defined in any of claims 2 to 22.

48. A prodrug according to claim 1, wherein said pharmacologically active compound is a pharmacologically active nucleic acid or a pharmacologically active fragment of a nucleic acid.

49. A technique according to claim 23, wherein said pharmacologically active compound is a pharmacologically active nucleic acid or a pharmacologically active fragment of a nucleic acid.

FIGURE 1: EFFECT OF PRODRUG ON CALCIUM LEVEL IN HUMAN LYMPHOCYTES



Axis:
X - Forward scattering
Y - Intracellular calcium level(au)
L- Low calcium level
H- High calcium level
Z - Events

FIGURE 2: RECOVERY IN GLOBAL CEREBRAL ISCHEMIA

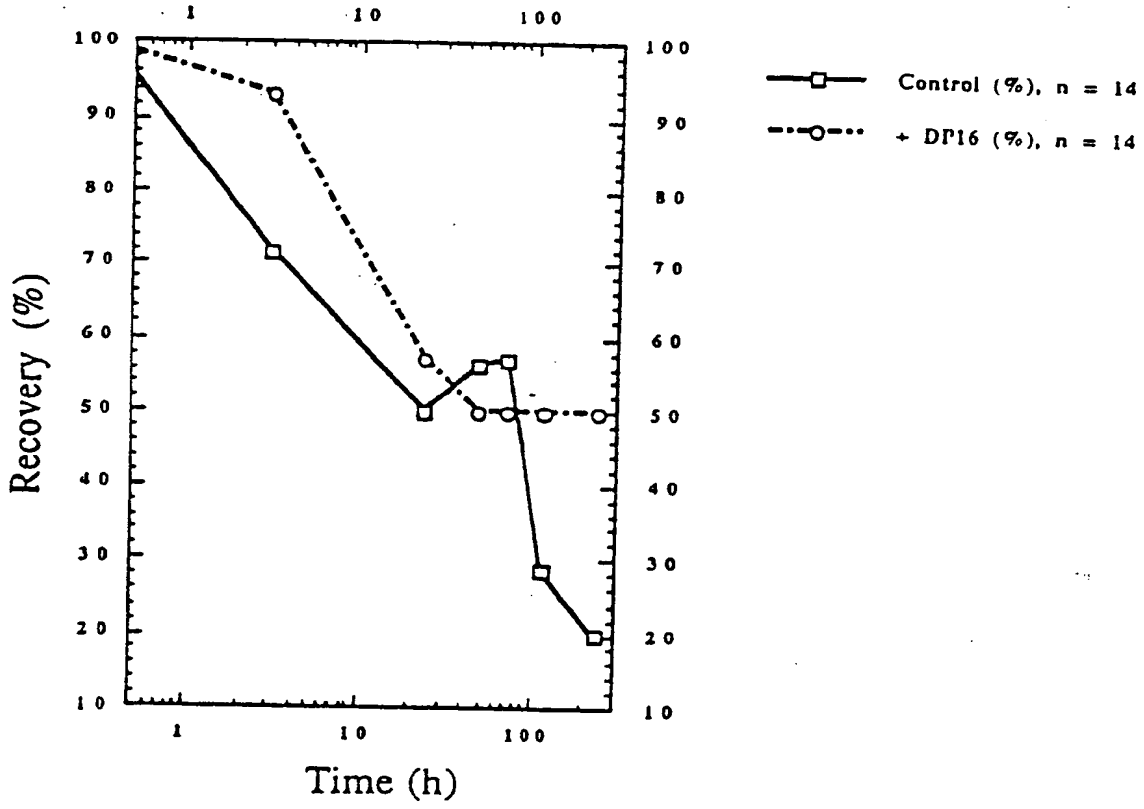


FIGURE 3: PILOCARPINE INDUCED EPILEPTIC EVENTS

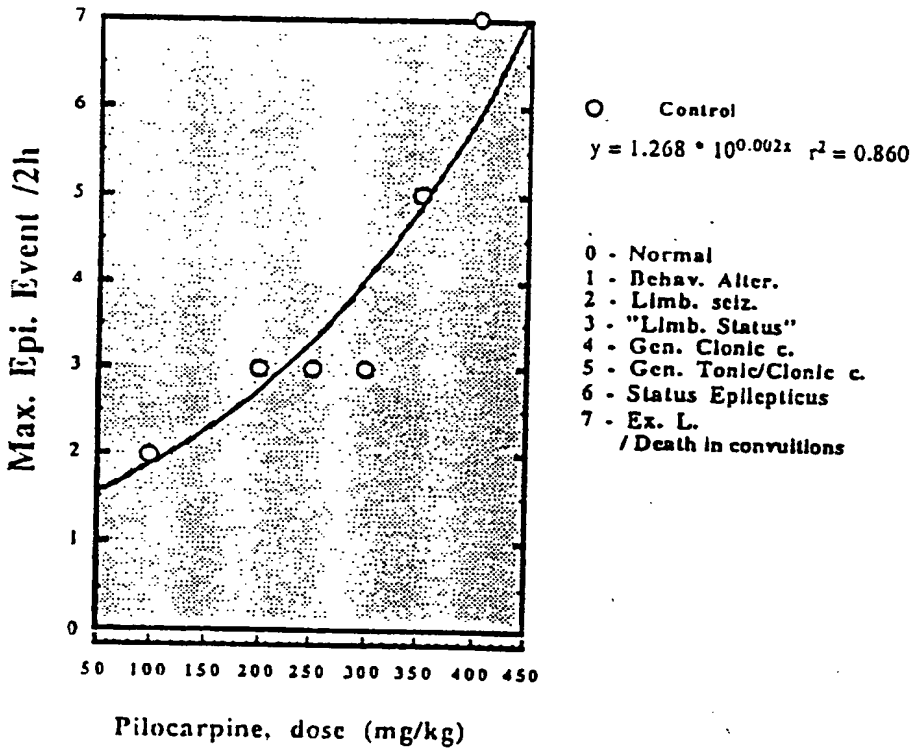


FIGURE 4: PROTECTION AGAINST PILOCARPINE INDUCED EPILEPTIC EVENTS

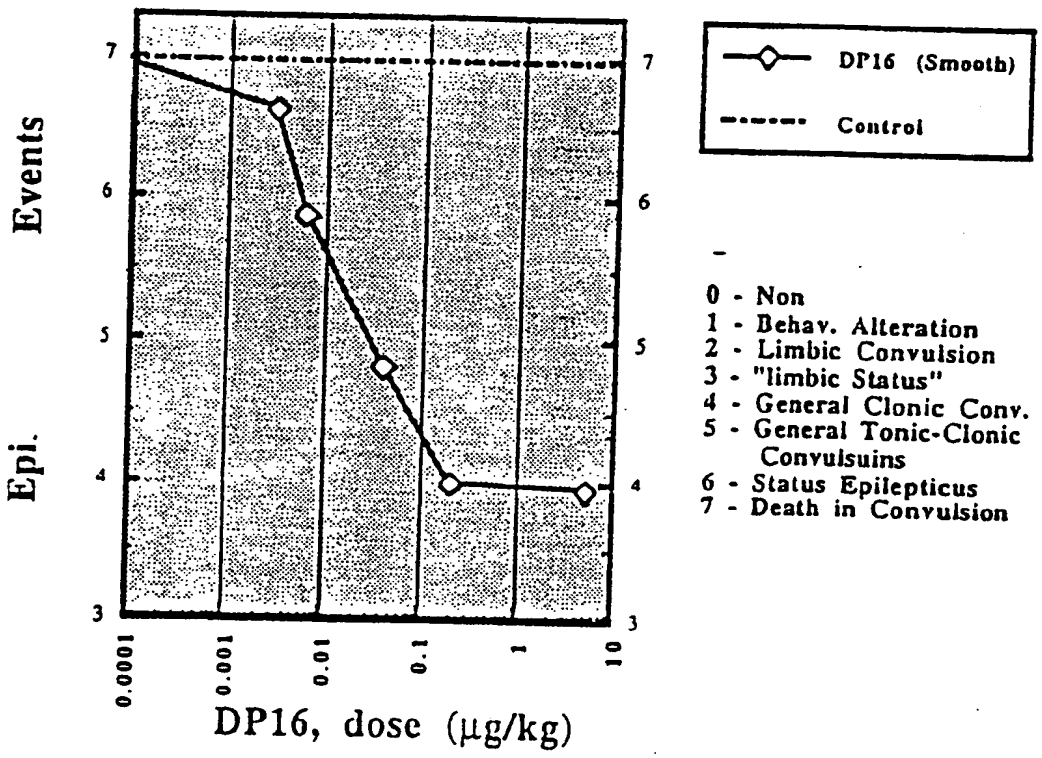
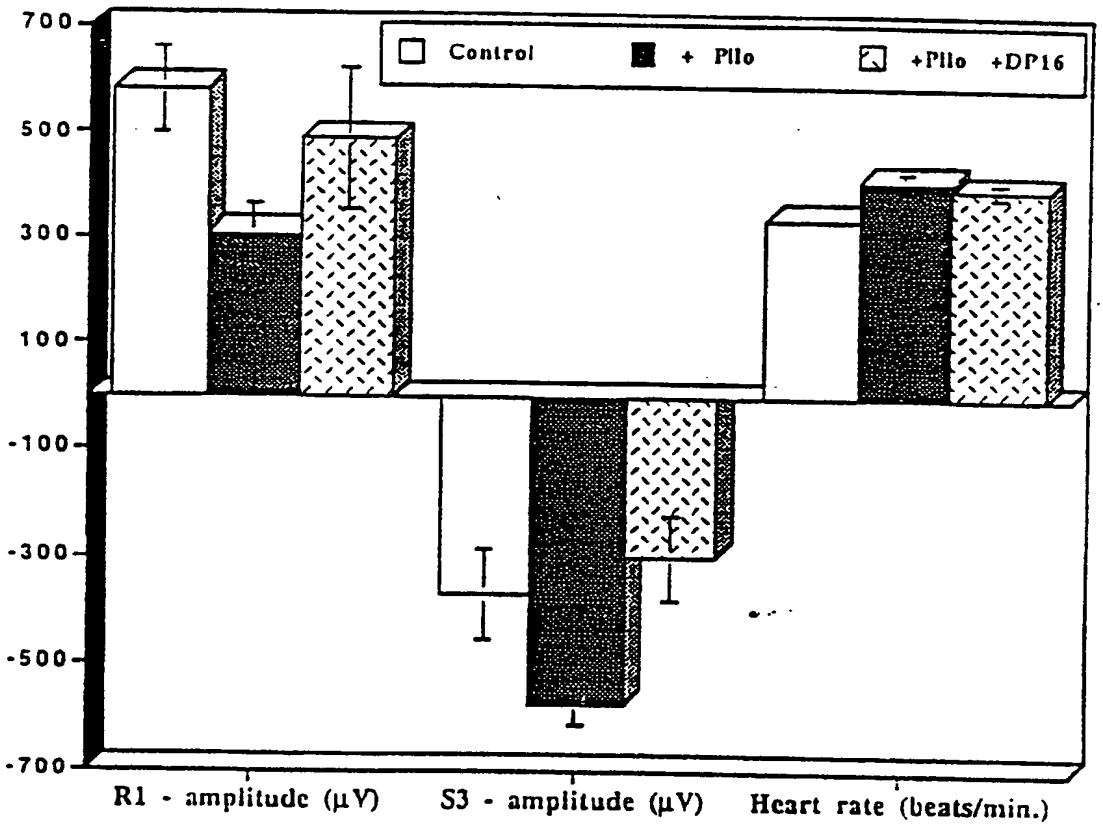


FIGURE 5: PROTECTION AGAINST PILOCARPINE INDUCED LONG-TERM ALTERATION OF CERTAIN CARDIAC FUNCTIONS



ECG Events

FIGURE 6: PROTECTION AGAINST PILOCARPINE INDUCED LONG-TERM SHIFT OF CORONARY VESSELS TONE REGULATION

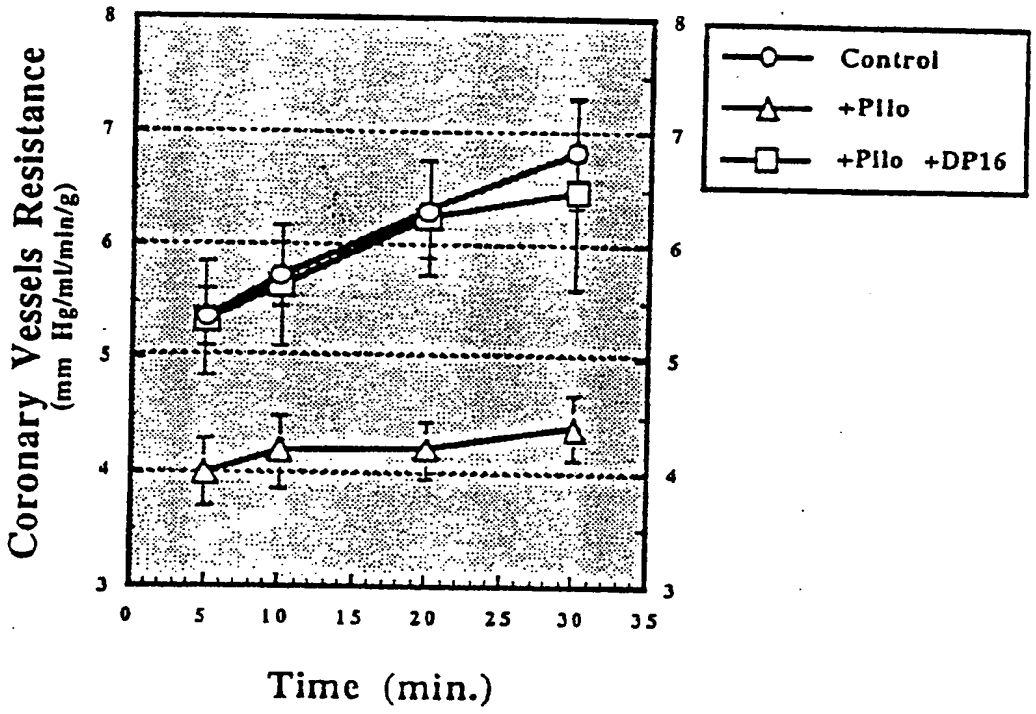


FIGURE 7: RECOVERY OF PILOCARPINE-DAMAGED HEARTS IN AN ISCHEMIA-REPERFUSION MODEL

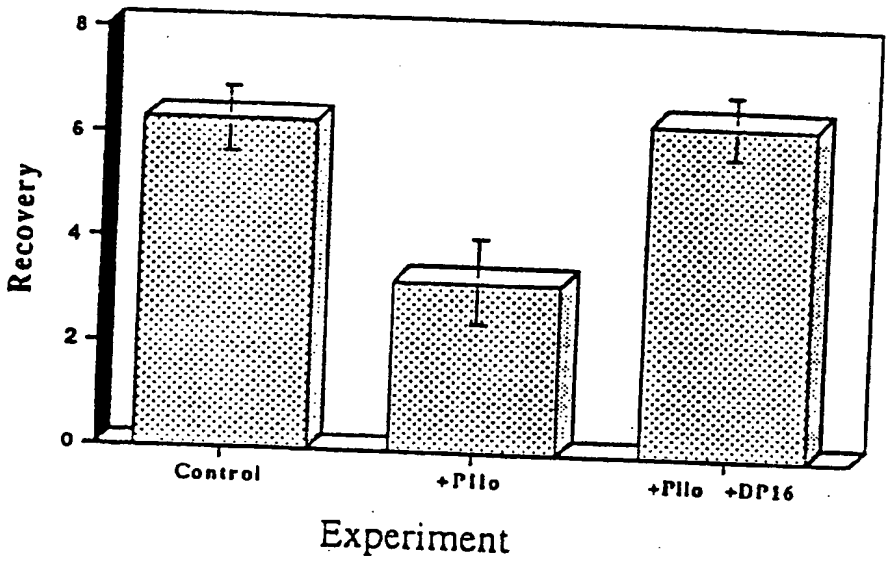


FIGURE 8: PROTECTION IN A METRAZOL MINIMUM SEIZURES TEST

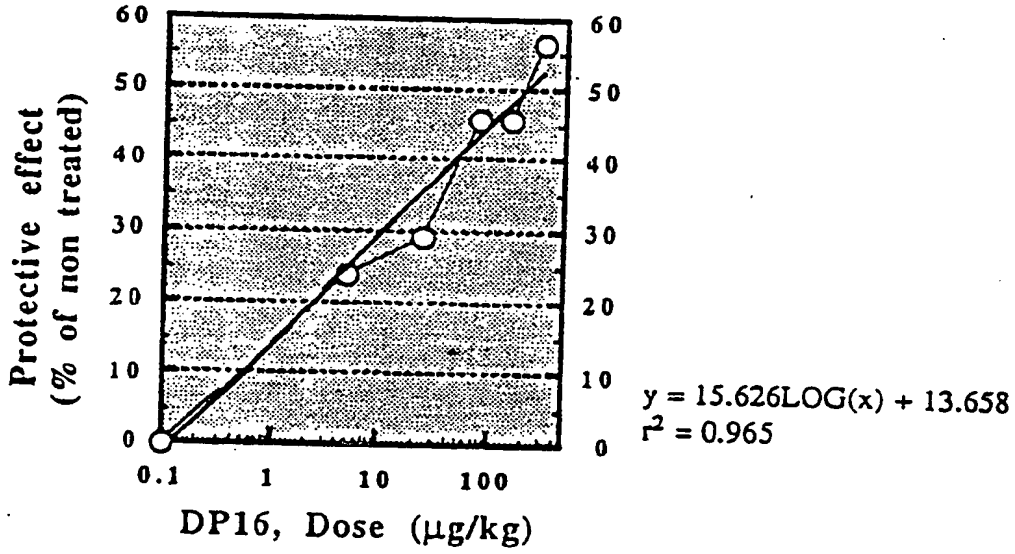
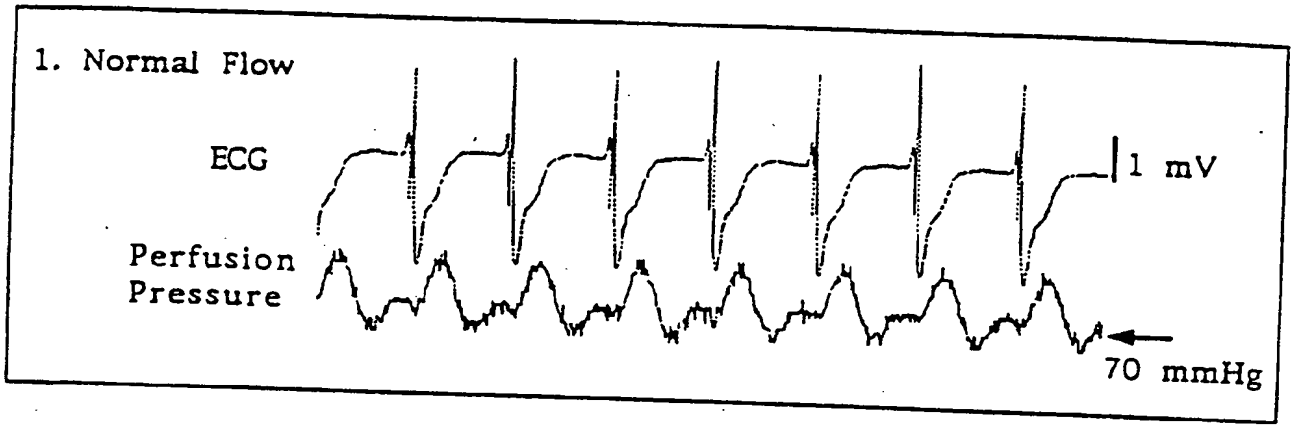
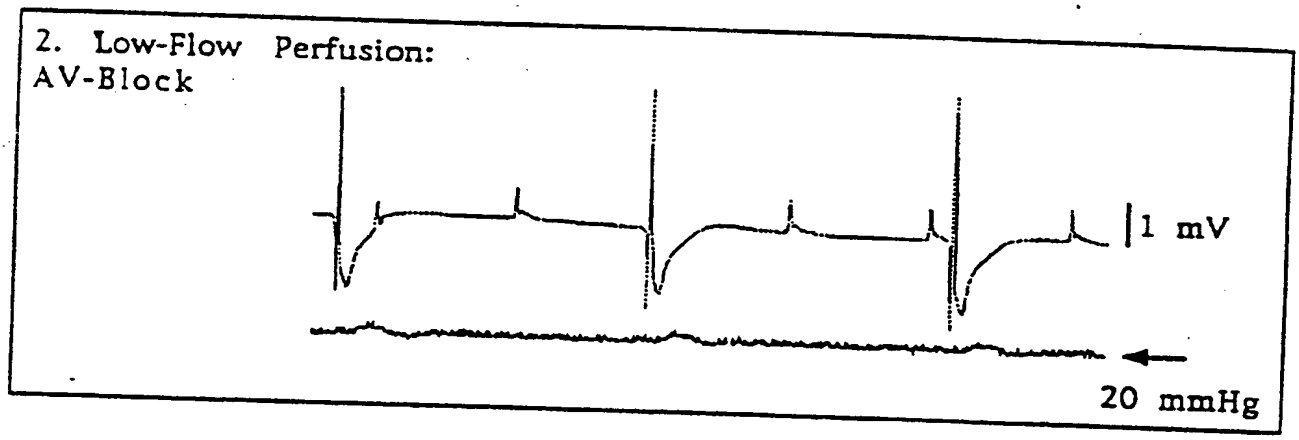


FIGURE 9: HYPOXIA-REPERFUSION CARDIOPATHOLOGY (CONTROL NO. 1)





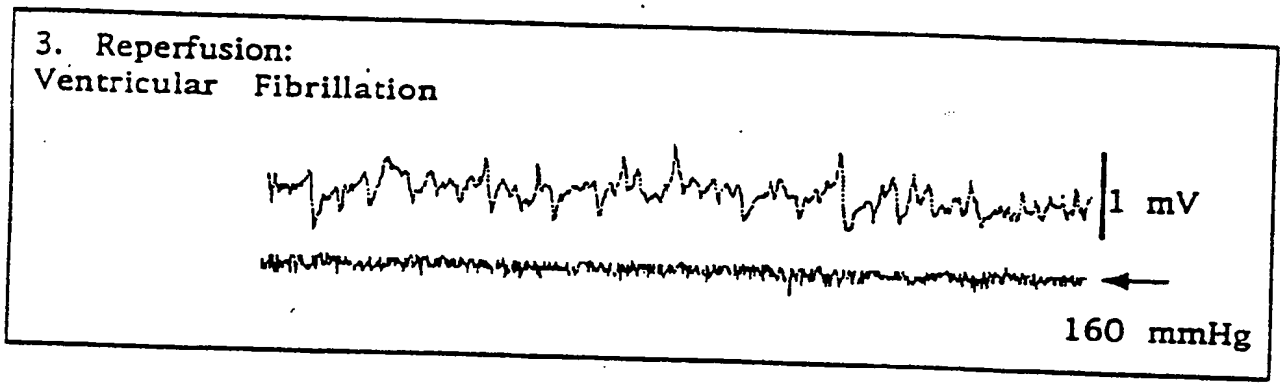


FIGURE 10: HYPOXIA-REPERFUSION CARDIOPATHOLOGY (CONTROL NO. 2)

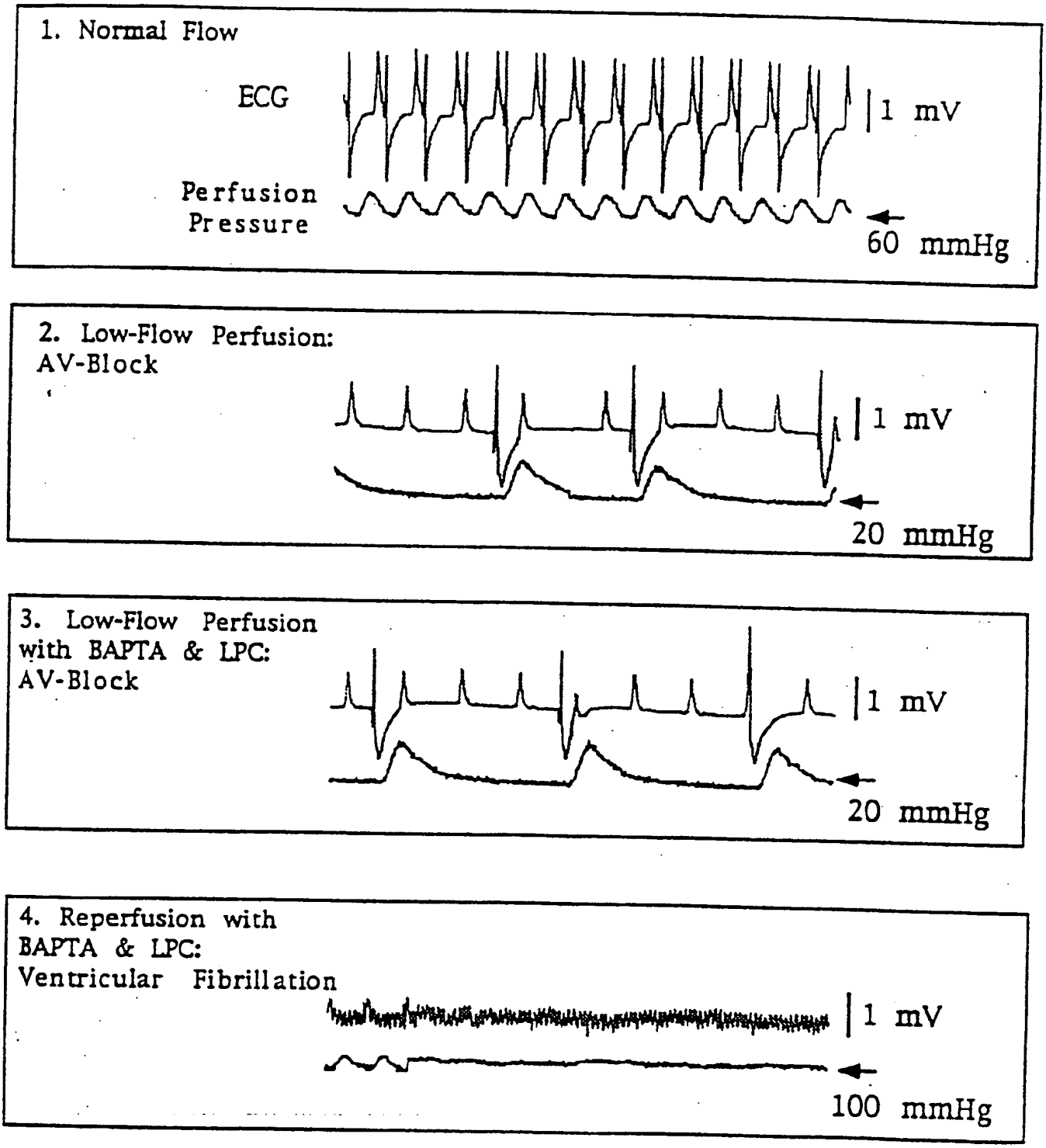
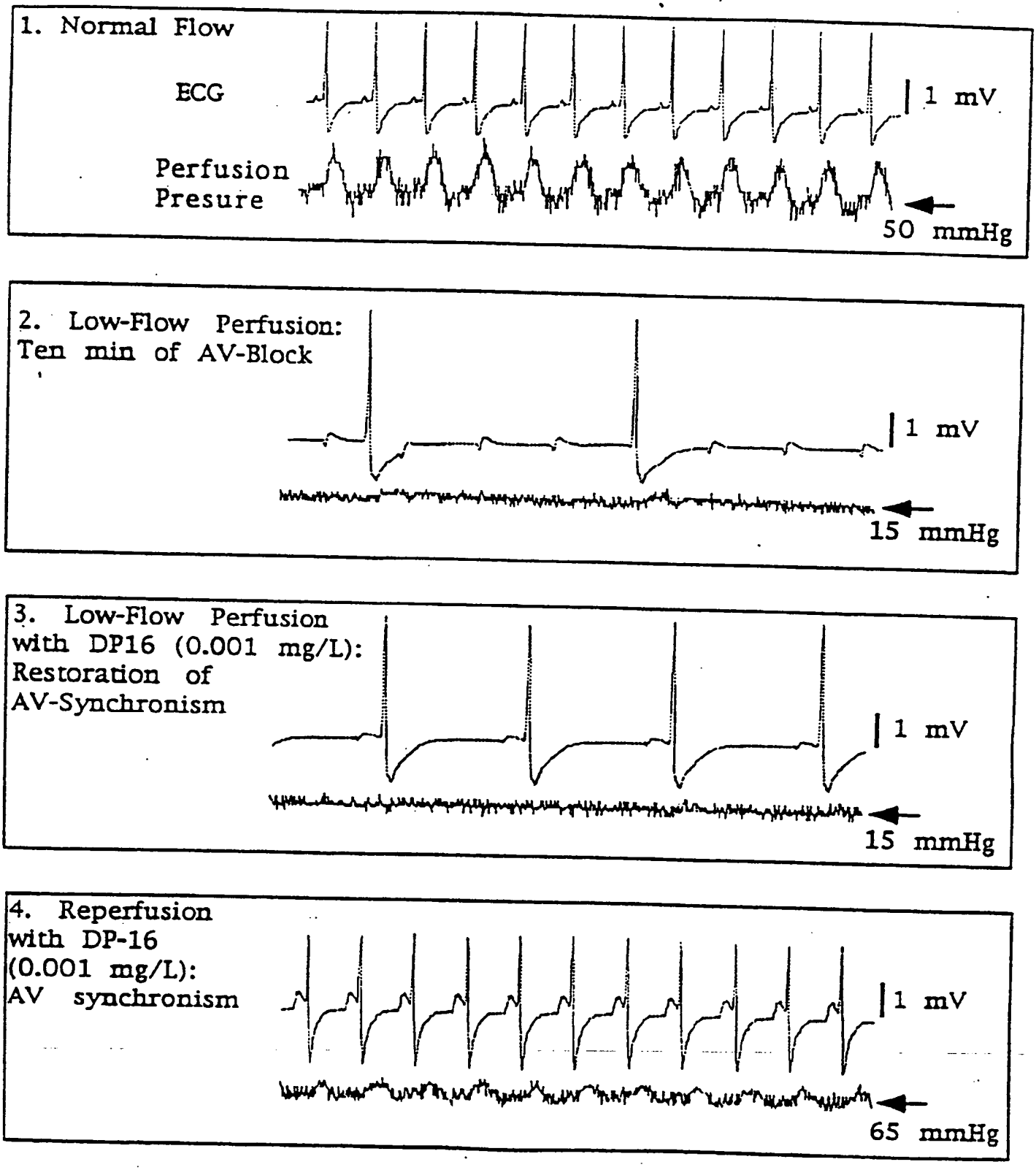


FIGURE 11: HYPOXIA-REPERFUSION CARDIOPATHOLOGY (TREATMENT)



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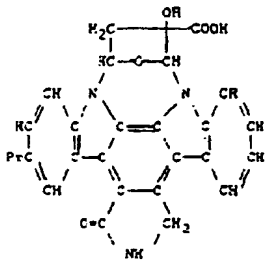
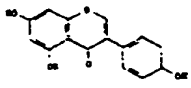
(88) Date of publication of the international search report:
22 December 1994 (22.12.94)

(54) Title: PRODRUGS WITH ENHANCED PENETRATION INTO CELLS



Protein kinase inhibitor K252b



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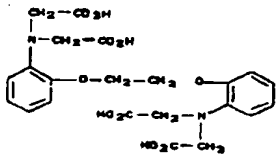

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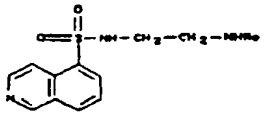

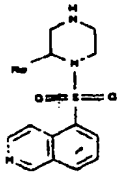

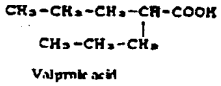

Valproic acid

(57) Abstract

The invention relates to a pharmaceutically acceptable prodrug which is a covalent conjugate of a pharmacologically active compound and an intracellular transporting adjuvant, characterized by the presence of a covalent bond which is scission-sensitive to intracellular enzyme activity. The prodrug may be used in a technique for treating a condition or disease in a human related to supranormal intracellular enzyme (e.g. phospholipase and/or esterase) activity, whereby on administering it to a human having such condition or disease, the bond is broken in response to such activity, and the pharmacologically active compound accumulates selectively within cells having such supranormal intracellular enzyme activity. Exemplary conjugates are esters of the carboxylic function in the formula, with e.g. heptanoyl-sn-3-glycerophosphoryl-choline or octanoyl-sn-3-glycerophosphoryl-choline.

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VN	Viet Nam

Internal Application No
PCT/GB 94/00669

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K47/48 C07F9/6561 C07F9/10 C07F9/62 C07F9/6558

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, vol.38, no.1, February 1992, STUTTGART DE pages 1 - 6 O. VAIZOGLU & P.P. SPEISER 'The pharmacosome(R) drug delivery approach' see abstract see introduction see paragraph 4.3 -paragraph 5 see figures see table 1 ---	1-14, 23-37, 46-49
X	EP,A,0 325 160 (HOECHST A.G.) 26 July 1989 see page 16, line 14 - line 23; examples --- -/--	1-14, 23-37, 46-49

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 1994	18. 11. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	DULLAART A.W.M.

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 05358 (UNIVERSITY OF IOWA RESEARCH FOUNDATION) 15 June 1989 see page 15; claims 9,14-16,22,24,25,28,29	1-14, 23-37, 46-49
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X	WO,A,90 10448 (GENENTECH INC) 20 September 1990 see examples	1-14, 23-37, 46-49
X	WO,A,93 00910 (VICAL, INC) 21 January 1993 see page 3, line 13 - line 34 see page 7, line 1 - page 8, line 20 see page 17, line 23 - line 32 see examples 1-5	1-14, 23-37, 46-49
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X	WO,A,91 16920 (VICAL, INC) 14 November 1991 see page 2, line 17 - page 3, line 23 see chemical formulae see examples see page 18 - page 19 see claims	1-14, 23-37, 46-49
X	WO,A,90 00555 (VICAL, INC) 25 January 1990 see examples	1-14, 23-37, 46-49

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	J PHARMACOL EXP THER, VOL. 252, NO. 2, PAGE(S) 466-73, February 1990 GUSOVSKY F ET AL 'Mechanism of maitotoxin-stimulated phosphoinositide breakdown in HL-60 cells.' see abstract see page 469 - page 470	1-14, 23-37, 46-49
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Y	J BIOL CHEM, VOL. 268, NO. 2, PAGE(S) 930-7, 15 January 1993 NATARAJAN V ET AL 'Activation of endothelial cell phospholipase D by hydrogen peroxide and fatty acid hydroperoxide.' see abstract see table 4 see page 936	1-14, 23-37, 46-49

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEBS LETT., VOL. 316, NO. 2, PAGE(S) 170-4, 1993 COORSSEN, JENS R. ET AL 'GTP.gamma.S and phorbol ester act synergistically to stimulate both calcium-independent secretion and phospholipase D activity in permeabilized human platelets. Inhibition by BAPTA and analogs' see page 170 see paragraph 3.3 -paragraph 4	1-14, 23-37, 46-49
E	WO,A,94 08573 (M.P. CHARLTON ET AL) 28 April 1994 see page 8, line 5 - line 23 see page 15, line 32 - page 18, line 10 see examples	1-14, 23-37, 46-49
T	BIOCHEM BIOPHYS RES COMMUN, VOL. 199, NO. 1, PAGE(S) 368-73, 28 February 1994 DUAN RD ET AL 'Conversion to Ca(2+)-independent form of Ca2+/calmodulin protein kinase II in rat pancreatic acini.' see abstract see page 369 see page 372	1-14, 23-37, 46-49

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/00669

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims: 2-14,24,37,48,49, and 1,23,46,47 in part.
2. Claims: 16,17,39,40, and 1,15,23,38,46,47 in part.
3. Claims: 18-20,41-43, and 1,15,23,38,46,47 in part.
4. Claims: 21,22,44,45, and 1,15,23,38,46,47 in part.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

2-14,24,37,48,49, and 1,23,46,47 in part

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Intern: al Application No
PCT/GB 94/00669

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